University of Valladolid

Faculty of Science

Department of Analytical Chemistry

Analysis of volatile compounds in bread and related products.

Improvement of gluten-free breads aroma.

Joana Pico Carbajo

Doctoral Thesis 2018

TESEA
Técnicas de separación y análisis aplicado

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PROGRAMA DE DOCTORADO EN QUÍMICA

Facultad de Ciencias
Departamento de Química Analítica

TESIS DOCTORAL:

ANALYSIS OF VOLATILE COMPOUNDS IN BREADS AND RELATED PRODUCTS. IMPROVEMENT OF GLUTEN-FREE BREADS AROMA.

Presentada por **Dª Joana Pico Carbajo** para optar al grado de **Doctora Internacional en Química** por la Universidad de Valladolid

Dirigida por:

Dr. José Bernal del Nozal
Dr. Manuel Gómez Pallarés

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La autora de la presente memoria ha disfrutado de un “Contrato Predoctoral de la Universidad de Valladolid cofinanciado por el banco Santander”, convocatoria 2013. Del mismo modo, ha disfrutado de una “ayuda para la realización de estancias breves en el desarrollo de tesis doctorales de la Universidad de Valladolid”, convocatoria 2015. Finalmente disfrutó también de las “ayudas para la asistencia a congresos durante el desarrollo de tesis doctorales de la Universidad de Valladolid” en sus convocatorias 2016 y 2017. Es por ello que expreso mi agradecimiento a esta entidad.

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“La humanidad también necesita soñadores, para quienes el desarrollo de una tarea sea tan cautivante que les resulte imposible dedicar su atención a su propio beneficio”

“Humanity also needs dreamers, for whom the disinterested development of an enterprise is so captivating that it becomes impossible for them to devote their care to their own material profit”.

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Foreword

This Doctoral Thesis is composed by a series of original research papers, according to the Spanish national (R.D. 99/2011, BOE 35/2011) and regional (BOCYL 243/2012) regulations.

Firstly, an extended introduction is presented for contextualisation of the topic and its relevance and for facilitating the insight of the state of art that gave rise to the publications included in this Doctoral thesis. Then, the exposition of the justification and objectives helps to understand the reasons that promote each specific research in a logical temporal sequence, taking into consideration the results of the previous study. Thereupon, the different sections disclose the 12 publications included in this Doctoral Thesis, which give exhaustive details of the work done. Likewise, the discussion of the results and conclusions summarise and interconnect the main findings obtained from the research carried out in order to achieve the specific and global objectives. Finally, an annex is included with the aim of showing other activities and merits that, doubtless, help to improve the quality of this research.

Prior to its defense, this Doctoral Thesis has been evaluated by two experts from international research centers directly related to its subject. The minor changes suggested by the following experts were performed:

* **Dr. Vittorio Capozzi**, Department of Agricultural Science, Food and Environment, University of Foggia (Italy).
*** Dr. Phil Bremer,** Department of Food Science, University of Ontago (New Zeland).

Abbreviations

AEDA (aroma extract dilution analysis)

BHT (butylated hydroxytoluene)

CAR (carboxen)

Cg (concentration in gas phase)

Cm (concentration in matrix)

CM (carboxymethyl cellulose)

DHS (dynamic headspace extraction)

DSC (differential scanning calorimeter)

DVB (divinylbenzene)

ECD (electron capture detector)

FD (flavour dilution factor)

FID (flame ionisation detector)

FOS (fructo-oligosaccharide)

FPD (flame photometric detector)

GC (gas chromatography)

GC×GC (comprehensive two-dimensional gas chromatography)

GC/O (gas chromatography/ olfactometry)

GPCRs (G-protein-coupled receptor)

HPMC (hydroxypropyl methyl cellulose)

HS (headspace)

IgE (immunoglobulin E)

IS (internal standard)

K (partition coefficient)

KI (Kovats index)

LAB (lactic acid bacteria)

LLE (liquid-liquid extraction)

LM (lipases method)

LRI (linear retention index)

MAHD (microwave-assisted hydro distillation)

MC (methyl cellulose)

MDGC (multidimensional gas chromatography)

MHE (multiple headspace extraction)

MS (mass spectrometry)

MSPD (matrix solid-phase dispersion)

NCGS (non-celiac gluten sensitivity)

OAV (odour activity value)

OT (odour threshold)

PA (polyacrylate)

PC (principal component)

PCA (principal component analysis)

PDMS (polydimethylsiloxane)

PEG (polyethylene glycol)

PFE (pressurised-fluid extraction)

ppm (parts per million)

PTR (proton-transfer reaction)

QTOF (quadrupole-time of flight)

RAST (radioallergosorbent)

RH (relative humidity)

RSD (relative standard deviation)

RVA (rapid visco analyser)

SAFE (solvent assisted flavour evaporation)

SDE (simultaneous steam distillation extraction)

SE (solvent extraction)

SEM (scanning electron microscope)

SFE (supercritical fluid extraction)

SHS (static headspace extraction)

SIDA (stable isotope dilution assay)

SIFT (selected ion flow tube)

SLE (solid-liquid extraction)

SM (SAFE method)

SPE (solid-phase extraction)

SPM (special packing material)

SPME (solid-phase microextraction)

SPT (skin prick test)

T (temperature)

TCD (thermal conductivity detector)

TOF (time of flight)

VS (vacuum sublimation)

WHO (world health organisation)

Resumen

El pan es uno de los alimentos naturales más consumidos a lo largo del mundo. De todas las propiedades que definen al pan, el aroma es una de las más importantes ya que determina la decisión del consumidor. La cromatografía de gases (GC) logra la separación y determinación individual de los compuestos volátiles, permitiendo entender mejor cómo diferentes procesos afectan a la formación de volátiles para poder así mejorar el aroma final del pan. Esta mejora del aroma del pan es aún de mayor importancia en los panes sin gluten, ya que hasta la actualidad han sido caracterizados por una baja calidad sensorial. El perfil aromático del pan se ve fuertemente influenciado por la harina y/o almidón empleado, ya que contiene los precursores que darán lugar a los compuestos volátiles. De este modo, la importancia del análisis de volátiles en pan no se basa solo en su control de calidad sino también en la mejora de sus características organolépticas.

Teniendo en cuenta todo esto, el principal objetivo de esta Tesis Doctoral ha sido la búsqueda de una receta a través de la cual el aroma del pan sin gluten sea mejorado mediante el empleo de las proporciones adecuadas de diferentes harinas y almidones sin gluten. Para ello, primero se alcanzaron diferentes objetivos intermedios. En primer lugar, se desarrolló un metodología de extracción con disolvente alternativa (método de las lipasas, LM) a la clásica metodología SAFE (SM) con la intención de mejorar principalmente su eficacia en la extracción y % RSD de precisión intermedia, a parte de la fragilidad y dificultad en la limpieza del aparato de destilación a vacío SAFE. El método LM consistió en una extracción durante 5 h a 40 °C con una mezcla dietiléter/ diclorometano (2:1) que contenía lipasas encargadas de hidrolizar la grasa convirtiéndola en glicerol y ácidos grasos, los cuales eluían al final del cromatograma. En segundo lugar, se estudió el tiempo máximo que una muestra de miga podría estar congelada, ya que los análisis de los aromas no siempre se podían realizar en el acto, lo cual llevaba a resultados poco precisos. Se concluyó que si el análisis se llevaba a cabo mediante extracción con disolvente, un máximo de una semana era recomendado, mientras que en el caso de SHS, se aconsejó el análisis en el mismo día de la preparación del pan. Finalmente, se estudió también la inhibición de la evolución fermentación para el análisis del aroma de las masas con una mezcla de octanoato de metilo y decanoato de metilo (Fames), como alternativa al $HgCl_2$ que presentaba elevada toxicidad, concluyéndose que la efectividad de la parada de la fermentación con Fames era cercana al 80 %. Disponibles todas las herramientas para llevar a cabo los análisis de aromas en panes, se prosiguió con el estudio del perfil aromático de panes sin gluten. En primer lugar se estudió, mediante LM, la evolución de los aromas en pan de almidón de maíz desde la masa a diferentes tiempos de fermentación hasta la miga horneada. Se llegó a la conclusión de que las masas estaban caracterizadas por compuestos de la fermentación y la miga, a parte de los compuestos de las masas, por compuestos de la oxidación de los lípidos. Dicha evolución se comprobó, mediante SHS-GC/MS, en masas y migas de panes sin gluten elaborados con almidón de maíz, de trigo y de patata y harinas de maíz (blanco y amarillo), arroz, avena, teff, quinoa, amaranto, trigo sarraceno y trigo (como control). De este estudio se eligieron las migas de los panes de quinoa, amaranto, teff, arroz y trigo (como control) y el almidón de maíz para examinar su perfil aromático por DHS-GC/MS, llegándose a las mismas conclusiones que por SHS-GC/MS: la harina de quinoa y el almidón de maíz fueron propuestos como las alternativas más adecuadas para la mejora del aroma de la miga debido a su elevado contenido en aromas agradables como el 3/2-metil-1-butanol y la 2,3-butanodiona y su bajo contenido en volátiles rancios como el hexanal y el 2,4-(E,E)-decadienal, lo cual provocaba además que sus perfiles aromáticos fueran más similares a los del pan de trigo. Puesto que el empleo de diferentes harinas y almidones sin gluten provocaba cambios en el perfil aromático de las correspondientes migas, se analizaron harinas de quinoa, teff, trigo sarraceno, arroz y almidón de maíz mediante SPME-GC/QTOF (método optimizado y validado) con la intención de ver si los propios volátiles de las bases harinosas se transferían directamente al pan elaborado. Los perfiles aromáticos obtenidos fueron completamente diferentes a los de las correspondientes migas, por lo que se concluyó que los volátiles debían de haber sido generados a través de los precursores de las harinas y almidones. En último lugar, se optimizó y validó un método semicuantitativo y cuantitativo SPME-GC/QTOF para el análisis de compuestos volátiles en cortezas de pan. Se seleccionaron las cortezas de harinas de teff, de arroz basmati, de arroz japonica y de trigo (muestra control) y de almidón de trigo para su cuantificación. Las cortezas fueron distinguidas principalmente por sus contenidos en pirazinas, 2-acetil-1-pirrolina, 2-(E)-nonenal y 2,4-(E,E)-decadienal, siendo las cortezas de teff y almidón de trigo las que mostraron un perfil más similar a la corteza de trigo. Así, las cortezas de teff y almidón de trigo fueron seleccionadas como las más adecuadas para la mejora del aroma de la corteza sin gluten tanto por su elevado contenido en pirazinas como por un contenido similar en 2-acetil-1-pirrolina y 4-hidroxi-2,5-dimetil-3(2H)-furanona con respecto a la corteza de trigo. Finalmente, seleccionadas la harina de quinoa y almidón de maíz como las mejores opciones para el aroma de la miga y la harina de teff y almidón de trigo como las mejores opciones para el aroma de la corteza, se propusieron mezclas de las mismas para mejorar el aroma final del pan sin gluten manteniendo buenas propiedades físicas, todo ello comparado con el pan de trigo (muestra control). Así, una mezcla 40 % almidón de maíz, 40 % almidón de trigo, 15 % harina de quinoa y 5 % harina de teff dio lugar a la menor proporción de aromas desagradables de la oxidación de los lípidos y la mayor proporción de alcoholes agradables de la fermentación, pirazinas y 2-acetil-1-pirrolina, mostrando además una de las cortezas más oscuras y una buena textura.

Abstract

Bread is one of the most commonly consumed staple foods around the world. Among all the properties that define bread, aroma is one of the most important, since it determines the bread's acceptability to the consumer. Gas chromatography (GC) allows for the separation and determination of individual volatile compounds, lending to better understanding of how different processes affect the generation of volatile compounds, and ultimately providing insight for ways to improve the final aroma of bread. Improvement of bread aroma is even more important in the case of gluten-free breads, since until now they have been characterised by a poor sensorial quality. The volatile profile is greatly influenced by the flour and/or starch employed, since they contain the precursors that lead to the formation of volatile compounds. Hence, the analysis of volatile compounds is important not only for promoting quality control but also for improving organoleptic characteristics.

Taking all into consideration, the main objective of this Doctoral Thesis has been the pursuit of a gluten-free bread recipe with improved aroma through the use of suitable proportions of different gluten-free flours and starches. For this purpose, specific objectives were firstly achieved. In the first place, an alternative solvent extraction methodology (lipases method, LM) to the classical SAFE methodology (SM) was developed with the aim of improving extraction efficiencies and the % of relative standard deviation (% RSD) of intermediate precision, apart from the fragility and the intricate cleaning of the SAFE device. The LM consisted mainly of an extraction for 5h at 40 °C with a mixture diethyl ether / dichloromethane (2:1) that contained lipases, which hydrolysed the fat into glycerol and fatty acids that eluted in the final part of the chromatogram. In the second place, the maximum freezing time suitable for a bread crumb sample was studied, since the aroma analyses cannot always be done instantly, leading to inaccurate results. It was concluded that, for a solvent extraction analysis, a maximum of one week of freezing was advised; meanwhile, in the case of SHS, analysis on the same day as bread preparation was recommended. Finally, in order to achieve accurate results in aroma analyses of the bread doughs, the effectiveness in fermentation inhibition with a mixture of methyl octanoate and methyl decanoate (Fames) was also studied as an alternative to the toxic $HgCl_2$. It was concluded that the effectiveness in the fermentation inhibition with Fames was close to 80 %.

Having all the tools for the analysis of volatile compounds in breads, the following tasks comprised the study of the volatile compounds of gluten-free breads. Primarily, the evolution of volatile compounds in corn starch bread from the dough at different fermentation times to the baked crumb was accomplished with LM. It was concluded that doughs were characterised by volatile compounds from fermentation. In the crumb, apart from the volatile compounds of the doughs, volatile compounds from lipids oxidation significantly contributed too. This evolution was also evaluated through SHS-GC/MS, in doughs and crumbs of gluten-free breads prepared with starches (corn, wheat and potato) and gluten-free flours (yellow corn, white corn, rice, oat, teff, quinoa, amaranth, buckwheat and wheat [as a control]). From this study, the crumbs from quinoa, amaranth, teff, rice and wheat (as a control) and corn starch were selected for volatile profile examination by DHS-GC/MS, reaching the same conclusions as with SHS-GC/MS: quinoa flour and corn starch were suggested as the most suitable options for the improvement of gluten-free crumb aroma due to the high content in pleasant aromas such as 3/2-methyl-1-butanol and 2,3-butanedione and the low content in rancid volatiles like hexanal and 2,4-(E,E)-decadienal, inducing volatile profiles similar to the wheat bread. Since the use of different gluten-free flours and starches led to changes in the volatile profile of the corresponding doughs, flours of quinoa, teff, buckwheat, rice and corn starch were analysed through SPME-GC/QTOF (optimised and validated method) with the aim of testing if the volatile compounds from the flour bases were transferred directly to the bread. The volatile profiles were completely different from the corresponding crumbs, indicating that the volatile compounds should have been generated through the precursors present in the flours and starches. Lastly, a semi-quantitative and quantitative SPME-GC/QTOF methodology was developed and validated for the analysis of volatile compounds in bread crusts. Teff flour, basmati rice flour, japonica rice flour and wheat flour (control sample) and wheat starch were selected for their quantification. Crusts were mainly distinguished by their content in pyrazines, 2-acetyl-1-pyrroline, 2-(E)-nonenal and 2,4-(E,E)-decadienal, with teff crust and wheat starch crust presenting the most similar volatile profiles to wheat flour crust (control sample). Thus, teff crust and wheat starch crust were selected as the most suitable options for the improvement of gluten-free bread crust both for its high content in pyrazines and also for presenting a similar content in 2-acetyl-1-pyrroline and 4-hidroxi-2,5-dimetil-3(2H)-furanone compared to wheat crust. Finally, once quinoa flour and corn starch were selected as the best alternatives for the improvement of the gluten-free crumb aroma and teff flour and wheat starch as the best alternatives for the improvement of the gluten-free crust aroma, different mixtures of these flours and starches were evaluated in order to improve the final aroma of gluten-free bread while maintaining good physical properties, with everything compared to wheat bread as a control sample. Thus, a mixture of 40 % wheat starch, 40 % corn starch, 15 % quinoa flour and 5 % teff flour gave rise to the lowest proportion of off-flavours from lipids oxidation and the highest proportion of pleasant alcohols from fermentation, pyrazines and 2-acetyl-1-pyrroline, showing one of the darkest crusts and a suitable texture.

List of original papers

Papers included in this Doctoral Thesis

Introduction

Paper 1: Pico, J., Bernal, J., & Gómez, M. (2015). Wheat bread aroma compounds in crumb and crust: A review. *Food Research International*, 75, 200-215. Impact factor (2016): 3.871.

http://dx.doi.org/10.1016/j.foodres.2015.05.051

Paper 2: Pico, J., Gómez, M., Bernal, J., & Bernal, J.L. (2016). Analytical methods for volatile compounds in wheat bread: A review. *Journal of Chromatography A*, 1428, 55-71. Impact factor (2016): 4.008.

http://dx.doi.org/10.1016/j.chroma.2015.09.045

Section 1

Paper 3: Pico, J., del Nozal, M. J., Gómez, M., & Bernal, J.L. (2016). An alternative method based on enzymatic fat hydrolysis to quantify volatile compounds in wheat bread crumb. Food Chemistry, 206, 110-118. Impact factor (2016): 4.498.

http://dx.doi.org/10.1016/j.foodchem.2016.03.054

Paper 4: Pico, J., Oduber, F., Gómez, M., & Bernal, J. Analytical feasibility of a SAFE method for aroma analyses in bread crumb. *Under Review*.

Section 2

Paper 5: Pico, J., Martínez, M. M., Bernal, J., & Gómez, M. (2017). Impact of frozen storage time on the volatile profile of wheat bread crumb. Food Chemistry, 232, 185-190. Impact factor (2016): 4.498.

http://dx.doi.org/10.1016/j.foodchem.2017.04.026

Paper 6: Pico, J., Bernal, J., del Nozal, M. J., & Gómez, M. (2017). Inhibition of fermentation evolution in bread doughs for aroma analyses. Flavour and Fragance Journal, 32, 461-469. Impact factor (2016): 1.644.

http://dx.doi.org/10.1002/ffj.3405

Section 3

Paper 7: Pico, J., Martínez, M. M., Bernal, J., & Gómez, M. (2017). Evolution of volatile compounds in gluten-free bread: From dough to crumb. Food Chemistry, 227, 179-186. Impact factor (2016): 4.498.

http://dx.doi.org/10.1016/j.foodchem.2017.01.098

Paper 8: Pico, J., Bernal, J. L., & Gómez, M. (2017). Influence of different flours and starches on gluten-free bread aroma. Journal of Food Science and Technology, 54, 1433-1441. Impact factor (2016): 1.597.

http://dx.doi.org/10.1007/s13197-017-2562-3

Paper 9: Pico, J., Hansen, Å.S., Petersen, M. A. (2017). Comparison of the volatile profiles of the crumb of gluten-free breads by DHE-GC/MS. Journal of Cereal Science, 76, 280-288. Impact factor (2016): 2.665.

http://dx.doi.org/10.1016/j.jcs.2017.07.004

Section 4

Paper 10: Pico, J., Tapia, J., Bernal, J., & Gómez, M. (2017). Comparison of different extraction methodologies for the analysis of volatile compounds in gluten-free flours and corn starch by GC/QTOF. Food Chemistry, *In press*. Impact factor (2016): 4.498.

http://dx.doi.org/10.1016/j.foodchem.2017.06.157

Paper 11: Pico, J., Antolín, B., Román, L., Gómez, M., & Bernal, J. (2018). Analysis of volatile compounds in gluten-free bread crusts with an optimised and validated SPME-GC/QTOF methodology. Food Research International, 106, 686-695. Impact factor (2016): 3.856.

https://doi.org/10.1016/j.foodres.2018.01.048

Section 5

Paper 12: Pico, J., Antolín, B., Román, L., Bernal, J., & Gómez, M. Selection of the most suitable mixture of flours and starches for the improvement of gluten-free breads through their volatile profiles. *Under Review*.

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Papers not included in this Doctoral Thesis

Paper 13: Martínez, M. M., Pico, J., & Gómez, M. (2015). Physicochemical modification of native and extruded wheat flours by enzymatic amylolysis. *Food Chemistry*, 167, 447-453. Impact factor (2015): 4.232.
http://dx.doi.org/10.1016/j.foodchem.2014.07.031

Paper 14: Martínez, M. M., Pico, J., & Gómez, M. (2015). Effect of different polyols on wheat and maize starches paste and gel properties. *Food Hydrocolloids*, 44, 81-85. Impact factor (2015): 4.703.
http://dx.doi.org/10.1016/j.foodhyd.2014.09.011

Paper 15: Pico, J., Martínez, M. M., Martín, M. T., & Gómez, M. (2015) Quantification of sugars in wheat flours with an HPAEC-PAD method. *Food Chemistry*, 173, 674-681. Impact factor (2015): 4.232.
http://dx.doi.org/10.1016/j.foodchem.2014.10.103

Paper 16: Martínez, M. M., Pico, J., & Gómez, M. (2016). Synergistic maltogenic a-amylase and branching treatment to produce enzyme-resistant molecular and supramolecular structures in extruded maize matrices. *Food Hydrocolloids*, 58, 347-355. Impact factor (2016): 5.459.
http://dx.doi.org/10.1016/j.foodhyd.2016.02.027

Paper 17: Pérez, M., Pico, J., Bernal, J., & Gómez, M. (2017) .Gluten free bread crust: Effect of protein enrichment. *Under review*.

Paper 18: Román, L., Pico, J., & Gómez, M. (2017). Extruded flours to improve the pick-up and crispness of fried coated batters. *Under review*

Paper 19: Pico, J., Khomenko, I., Capozzi, V., Navarini, L., Bernal, J., Gómez, M., & Biasioli, F. (2017). Analysis of volatile compounds in different gluten-free bread baked and roasted crumbs and crusts by direct PTR/MS and fast-GC- PTR/MS. *Under review*.

Paper 20: : Pico, J., Khomenko, I., Capozzi, V., Navarini, L., Bernal, J., Gómez, M., & Biasioli, F. (2018). Understanding the generation of volatile compounds during baking and roasting: on-line monitoring of gluten-free breads aroma by PTR-ToF-MS. *Under review*.

Paper 21: Pico, J., Khomenko, I., Lonzarich, V., Navarini, L., & Biasioli, F. (2018). Model systems for examining the generation of volatile compounds during Maillard reaction and caramelisation by PTR-ToF-MS, GC/MS and sensorial analyses. *Under review*.

Paper 22: Pico, J., del Nozal, S., Bernal, J. & Bernal, J. L. (2018). Study of MS/MS transitions of volatile compounds by GC/QTOF: a database for food and beverages aroma analyses. *Under review*.

Contents

I. Introduction

** The introduction gave rise to two reviews, one regarding the volatile compounds commonly present in wheat bread and the other related to the analytical techniques employed in the analysis of bread aroma. These reviews were the result of an intensive searching and handling of information developed during the Doctoral Thesis. Nevertheless, the introduction has not been exclusively based on these two reviews, but certain parts were extracted and adapted to the structure of a Doctoral Thesis.*

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Review

Wheat bread aroma compounds in crumb and crust: A review

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Review article

Analytical methods for volatile compounds in wheat bread

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1. Introduction

Wilhelm Ostwald, Nobel prize in Chemistry (1909), defined Analytical Chemistry as "the art of separating, recognizing different substances and determining the constituents of a sample" (Gallo & Ferranti, 2016). Over the years, it has been applied to food analysis, belonging to a branch of chemistry called "food chemistry". Food can be defined as the material which, in its naturally occurring, processed or cooked form, is consumed by humans as nourishment and enjoyment (Belitz, Grosch, & Schieberle, 2009). The terms "nourishment" and "enjoyment" result in the nutritional and hedonic properties of food. The nutritional values are easy to determine, but the hedonic properties are trickier, including visual appeal, smell, taste and texture, which interact with the senses. Moreover, food chemistry not only analyses the composition of the raw materials and end-products, but also the changes occurred during food processing, storage and cooking. In this context, the highly complex nature of food results in a great number of desired and undesired reactions which are controlled by a variety of parameters. As a result, one of the main aims of food chemistry has been the application and development of analytical methods (Belitz et al., 2009).

1.1. Flavour and the process of smelling

Food quality depends on physical (e.g., appearance, colour, texture, viscosity) and chemical factors (e.g., vitamins, minerals, fibres, proteins, sugars, lipids). In this way, food preference is highly related to the stimulation of the human senses. Particularly, flavour takes the first place among the different factors that affect the quality of food (Rothe, 1988). Flavour is the overall sensation of taste and aroma/ odour (Belitz et al., 2009), where the taste is constituted by non-volatile compounds at room temperature and the aroma by volatile compounds from different chemical groups. Taste is sensed in the tongue through five taste sensations (sourness, sweetness, bitterness, saltiness and umami), while aroma is detected by the odour receptor sites of the smell organ, including in the nose (orthonasal detection) and the throat after being released by chewing (retronasal detection) (Belitz et al., 2009; Picó, 2012). Moreover, sometimes a single compound can be responsible for the taste and aroma at the same time.

It is widely accepted that the aroma is the most important characteristic of food flavour and most of the analytical researches have been focused on the aroma fraction (Picó, 2012). In fact, the consumers' acceptance, and therefore, the commercial values of food products depend considerably on the composition and amount of aroma substances (Cserháti, 2010). Wagner et al. (2014) studied the relationship between the hedonic responses to given food odours and the effective liking and disliking of food. They concluded that the food bearing stronger unpleasant flavours are perceived as potentially harmful by the olfactory system, which acts as a warning system against the intake of intoxicants. Therefore, it can be accomplished that the aroma of food is really important for the choice of the consumer.

Smell begins in the olfactory epithelium, a tissue located in the back of the nose and constituted by millions of sensory neurons, as it can be seen in **Figure 1**. The tips of the olfactory cells contain proteins called olfactory or odorant receptors (G-protein-coupled receptors, GPCRs) that bind the volatile compounds (Araneda, Kini, & Firestein, 2000). Each receptor can be activated by different molecules and each molecule can active different receptors, allowing the detection of a wide variety of smells. Once the molecule interacts with the olfactory receptor, an electrical signal is initiated travelling from the sensory neurons to the olfactory bulb that is located in the forebrain and relays the signal to other brain areas. One is the piriform cortex, whose function is the identification of the smell; the other is the thalamus, which serves as a relay station for the rest of brain areas of the sensory information, like the hippocampus and amygdale involved in learning and memory (Araneda et al., 2000; Buck & Axel, 1991; Bushdid, Magnasco, Vosshall, Keller, & Mixture,2016).

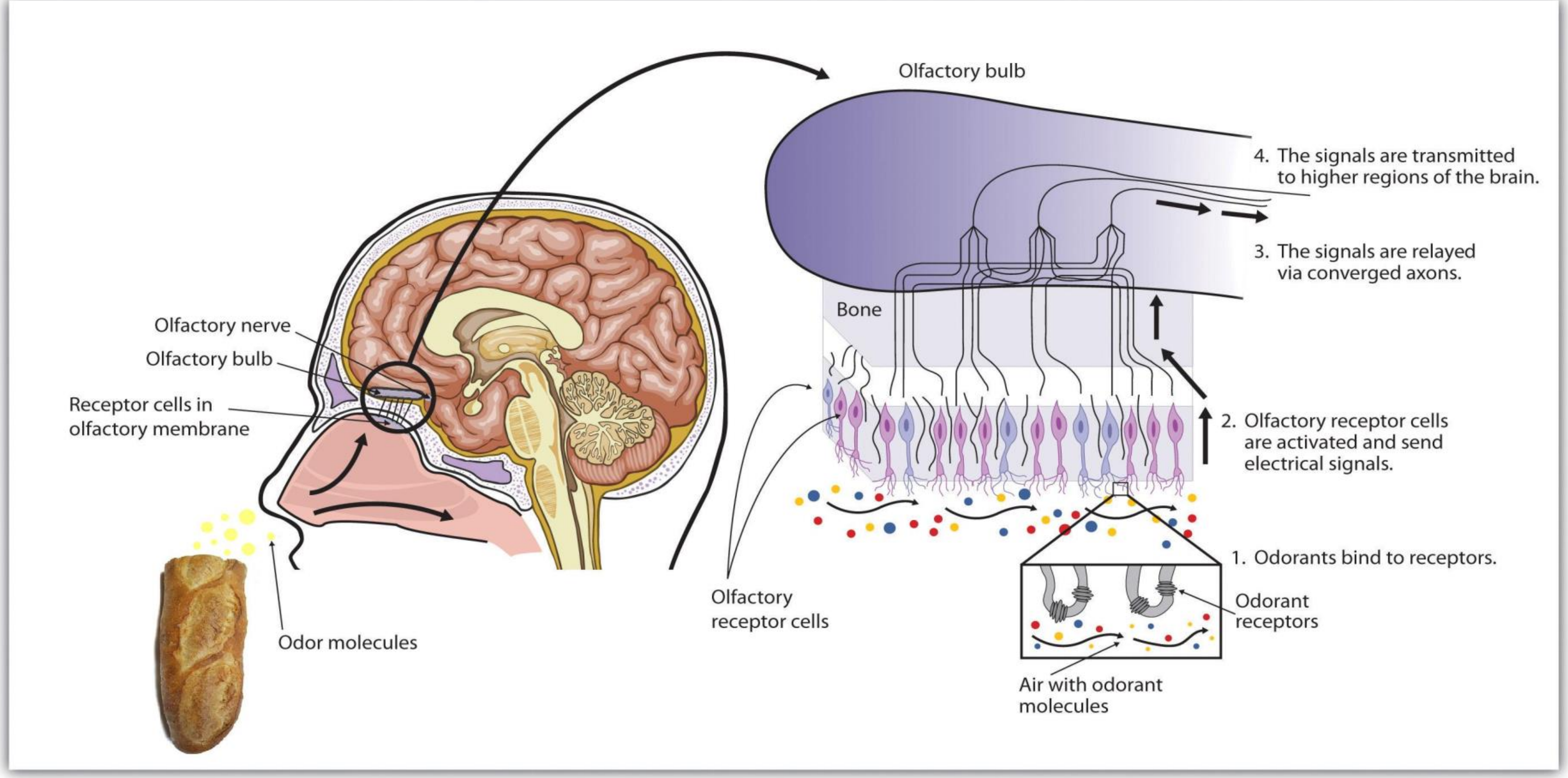

Fig.1. *Human body areas involved in the process of smelling (adapted from* http://open.lib.umn.edu/intropsyc/chapter/4-4-tasting-smelling-and-touching/)

In this way, normal adults can distinguish between nearly 2000 odour impressions and a trained panellist up to 10000 (Rothe, 1988).

1.2. Analysis of food aroma

Aroma research started more than seven decades ago, around 1946, when it was a domain of flavour industry with the use of flavouring agents in sweets, ice creams, beverages, confectionary as well as spices and seasonings for cooked dishes (Rothe, 1988). Flavour research has usually started with sensory analyses and it has been complemented or even replaced by the chromatographic analyses. The replacement of the sensory analyses by instrumental analyses has been only possible when the correlation between both data was known. The most common sensory methods have been the "ranking test", the "scaling test", the "sensory profile analysis" and the "acceptance test".

Nevertheless, without any doubt, the introduction by Martins and James in 1952 of gas-chromatography (GC) was the reason for the great development of the field of flavour research. Important landmarks that have given birth to the modern GC, employed in food aroma, have been summarised in **Table 1** (Rothe, 1988; Zellner, Dugo, Dugo, & Mondello, 2012). In this way, at the begging of 1907s less than 1500 volatile compounds had been identified in food products, while nowadays more than 7000 volatile compounds are known (Rowe, 2005).

Table 1. *Landmarks that have given birth to the modern GC*

Landmark	Developers	Year
Use of capillary chromatographic columns	Golay and Dukstra	1958
Introduction of headspace techniques	Buttery and Teranishi	1961
Introduction of gradient programs in the GC oven	Dalnogare, Bennet, Harrias and Habgood	1958-1966

Table 1. *(continued)*

Landmark	Developers	Year
Development of flame ionisation detector (FID)	Mcwilliam, Dewar and Harley	1957-1958
Development of electron capture detector (ECD)	Lovelock and Lipsky	1960
Development of flame photometric detector (FPD)	Brody and Chaney	1966
Establishment of the fast-GC	Desty	1962
Use of organic polymers as stationary phase	Hollis	1966
Coupling of GC with mass spectrometry (MS)	Yhage	1964
Development of the GC-Olfactometry (GC-O)	Fuller	1964
Apparition of Heart-Cutting Multidimensional GC (heart-cutting MDGC)	Deans	1968
Apparition of Comprehensive Two-Dimensional GC (GCxGC)	Liu and Phillips	1991

1.2.1. Analytical methods for volatile compounds in food

The concentration of volatile compounds in food is, generally, extremely low (Belitz et al., 2009; Cserháti, 2010; Zellner et al., 2012). Moreover, food matrices are quite complex and the volatile compounds need to be carefully separated from the non-volatile matrix. Then, it is absolutely necessary a sample preparation step that concentrates and purifies the analytes in order to decrease the influence of the matrix. Sample treatments in aroma analyses are very tricky due to the great variety of chemical classes that leads to differences in their volatility and instability. Moreover, food components can easily undergo to reactions during the isolation process (Zellner et al., 2012) and foods with active enzymes can suffer also aroma changes (Belitz et al., 2009). The possible changes that the volatile profile can suffer during aroma isolation are summarised in **Table 2** (Belitz et al., 2009).

There have emerged mainly three options in the analysis of volatile compounds in food: solvent extraction (SE), supercritical fluid extraction (SFE) and headspace analysis (HS). Once the analytes have been isolated from the matrix, they can be determined by GC with different detectors or by Proton Transfer Reaction/MS (PTR/MS). The selection of the analytical method depends on the food matrix and on the requirements of the research. The amount of sample, the time of sample treatment, the temperature of extraction or the use of environmentally friendly methods have been some of the main requirements taken into consideration when choosing an analytical methodology. It can be concluded that, in general, the choice of the method depends on the necessity: if a quick analysis is needed, HS or

Table 2. *Possible changes in food aroma during the isolation of volatile compounds*

Changes in food aroma during aroma isolation	
Enzymatic	**Non-enzymatic**
• Hydrolysis of esters • Oxidative cleavage of unsaturated fatty acids • Hydrogenation of aldehydes	• Hydrolysis of glycosides • Lactones from hydroxy acids • Cyclisation of di-, tri-, and polyols • Dehydration and rearrangement of tert-allyl alcohols • Reactions of thiols, amines, and aldehydes • Reduction of disulfides by reductones from the Maillard reaction • Fragmentation of hydroperoxides

SFE would be the best option, while if an accurate and wide volatile profile is preferred, SE would be the best solution. Therefore, it could be concluded that SE and HS methods should really be considered as complementary methodologies in the analysis of volatile compounds in food. SE and SPME have frequently been used in a complementary manner in order to obtain a complete profile of the overall flavour compounds (Corral, Salvador & Flores, 2015; Klensporf & Jeleń, 2008; Majcher & Jeleń, 2009; Thompson-Witrick et al., 2015; Wang, Song, Zhang, Tang & Yu, 2016). Solvent extracts have reportedly been richer in high-molecular weight volatile compounds, while SPME extracts have been richer in low-molecular weight volatile compounds (Mayuoni-kirshinbaum, Tietel, Porat, & Ulrich, 2012), which is very important for those compounds that are very volatile and co-elute with the solvent (Machjer & Jeleń, 2009).

1.2.1.1. Solvent extraction (SE) methodologies

SE methodologies have usually yielded complete volatile profiles, which are not necessarily representative of the aroma of the sample (Zellner et al., 2012). It has entailed the use of organic solvents and, therefore, it has been useful for fat-free foods or foods with a very low content in fat, since the fat is easily extracted with the organic solvent. In the case of liquid samples, it has consisted in a liquid-liquid extraction (LLE) normally followed by an isolation of the volatile compounds from the matrix. For solid matrices, it has consisted in a solid-liquid extraction (SLE) and it has been the extract which has been submitted later to an isolation of the volatile compounds from the extracted matrix. The extraction has been performed with the conventional agitation using a separating funnel (liquid sample) or a shake flask (solid sample), although the most common practise has been the use of a Soxhlet apparatus (Cserháti, 2010; Pico, Gómez, Bernal, & Bernal, 2016). As high amounts of sample and solvents are needed, it is usually necessary to reduce the volume up to 500 µL or less before GC/MS analyses, and for this purpose a Vigreux column is employed (Kirchhoff & Schieberle, 2001; Moskowitz, Bin, Elias, &

Peterson, 2012; Rychlik & Grosch, 1996). The most common solvent has been dichloromethane (Engel, Bahr, & Schieberle, 1999; Gassenmeier & Schieberle, 1995; Schieberle & Grosch, 1994; Zehentbauer & Grosch, 1998a), although diethyl ether has been also employed for acidic compounds (Zehentbauer & Grosch, 1998a).

If the amount of non-volatile compounds is high, mainly fat, it could contaminate the GC liners and columns (Zellner et al., 2012). In order to isolate the volatile compounds from the non-volatile fraction, distillation or sublimation techniques have normally been used. Three alternatives have so far been reported (Pico, Gómez, et al., 2016), showed in **Figure 2**: Simultaneous Steam Distillation Extraction (SDE) (Lin, Hsieh, Liu, Lee, & Mau, 2009), Vacuum Sublimation (VS) (Rychlik & Grosch, 1996; Schieberle & Grosch, 1991) and Solvent Assisted Flavour Evaporation (SAFE). The most common employed nowadays is SAFE, which was developed in 1999 by Engel, Bahr and Schieberle (1999). The extract coming from the SLE or the liquid sample is placed in the dropping funnel (number 1). The distillation flask (number 2) is heated in a water bath between 20 and 30 °C and the head (number3) and legs (number 8 and 9) should be thermostated at the same temperature, in order to avoid the solvent freezing. Liquid nitrogen is poured into the cooling trap (number 7) and into the safety cooling trap (number 5). When the high vacuum is applied, aliquots of the sample (10 mL/min of oily samples) are dropped from the dropping funnel into the distillation flask. The vapours which contain both volatile compounds and solvent, rises along the left leg to the head, where propeller-shaped barriers remove the non-volatile materials. Then, the vapours down along the right leg and condensed in cooled flask (number 4).

The main drawbacks of SE methods include the possible losses of the more volatile compounds during solvent removal, the need of large sample amounts as well as the possibility of co-elution between the solvent peak and the early-eluting aroma compounds (Zellner et al., 2012).

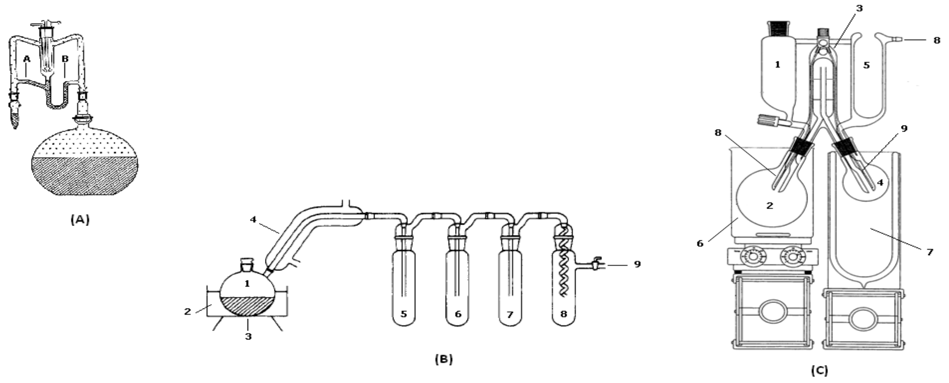

Fig.2. *Schemes of different distillation alternatives employed in volatile fraction isolation.* ***(A)*** *Simultaneous Steam Distillation Extraction equipment,* ***(B)*** *Vacuum Sublimation equipment,* ***(C)*** *Solvent Assisted Flavour Evaporation equipment (from Pico et al., 2016).*

1.2.1.2. Supercritical fluid extraction (SFE) methodologies

SFE is a promising alternative to the common solvent extraction, which usually requires the use of organic solvents. Environmentally friendly mobile phases are employed, namely, carbon dioxide or carbon dioxide mixed with organic solvents (Cserháti, 2010). The most employed supercritical fluid is carbon dioxide, which extraction conditions are above the critical temperature (31°C) and critical pressure (74 bar), acting as a non-polar solvent. Modifying the pressure could make possible to increase the selectivity of the extraction, avoiding the extraction of lipids (Seitz, Ram, & Rengarajan, 1999).

1.2.1.3. Headspace extraction (HS) methodologies

HS is the volume of vapour or gas formed above a solid or a liquid (matrix) when it is heated within a sealed vial, establishing equilibrium between the molecules that have escaped to the gas phase and the matrix. HS methodologies combine simplicity, solvent-free procedures, the need of small sample amounts, and no artifact formation, becoming more preferred in the analysis of food aroma than SE methodologies. In spite of their advantages, the non-volatile compounds can influence the partitioning of the analyte in the equilibrium (Fabre, Aubry, &

Guichard, 2002) and, moreover, the relative concentration of volatile components in the HS does not correspond to the concentration in the sample (Zellner et al., 2012). The partition coefficient (K) is temperature-dependent (**equation 1**) and provides an idea of the easy of the analyte to pass to the gaseous phase (**equation 2**).

$\frac{dK}{dT} = \frac{1}{T^2}$ (**equation 1**, K = partition coefficient, T = temperature)

$K = \frac{Cm}{Cg}$ (**equation 2**, Concentration in the matrix, C_m, and in the gas phase, C_g)

HS methodologies have commonly been divided into SHS, DHS and SPME (Belitz et al., 2009), although P&T has also been employed in the analyses of food aroma (Zellner et al., 2012). SHS, DHS and SPME could operate in Multiple Headspace Extraction (MHE) mode, which allows the quantification of the aroma compounds regardless of the food matrix (Birch, Petersen, & Hansen, 2013).

SHS has been the simplest option, since it injects directly into the GC a fraction of the HS. The main parameters to be controlled are the extraction time and temperature, the amount of sample and the shaking. Although it is a simple and cost-effective method, it presents low sensitivity and fails in the analysis of trace components or very high boiling point compounds, due to the saturation of the equilibrium.

DHS enhances the problem of sensitivity of SHS, because the headspace is constantly renewed by purging the volatile compounds with an inert gas and concentrating them in a trap, which is later desorbed in the injection port of the GC (Pico et al., 2016; Zellner et al., 2012). However, DHS analyses the ratio of each volatile compound concentrated in the trap but not the direct measurement of the gaseous phase, as SHS does (Maeda et al., 2009). The parameters to be controlled are the same than for SHS adding the inert gas, the sorbent trap (usually the polymer Tenax TA ©) and the thermal desorption conditions.

SPME, developed by Arthur and Pawliszyn (1990), is based on the partitioning of the analytes between the extracting phase, immobilised on a fused silica fibre, and the matrix. Once the equilibrium is reached, the adsorbed/absorbed compounds are thermally desorbed in the injection port of the GC (Xu et al., 2016). The volatile compounds can be absorbed or adsorbed depending on the extracting phase: CAR/PDMS (carboxen /polydimethylsiloxane), PDMS/DVB (polydimethylsiloxane/ divinylbenzene) and DVB/CAR/PDMS (divinylbenzene/ carboxen/ polydimethylsiloxane) perform by adsorption, which depends mainly on the particle size, forcing the analytes to compete for the sites. However, PDMS (polydimethylsiloxane), PA (polyacrylate) and PEG (polyethyleneglycol) perform by absorption, which strongly depends on the polarity of the analytes, PDMS being suitable for unpolar compounds and PA and PEG for polar compounds. It has become in one of the preferred options, although it also requires the precise control of the extraction time and temperature, the fibre coating as well as the desorption conditions. As for DHS, SPME analyses the ratio of each volatile compound concentrated in the fibre, which depends on the type, thickness, and length of the fibre.

1.2.1.4. Electronic nose and electronic mouth and other possibilities in the sample treatment of food aroma

The electronic nose is a device with detectors of odours based normally in chemical gas sensors (commonly metal oxide sensors), although new approaches have emerged, like optical sensors, mass spectrometric detectors or infrared detectors (Röck, Barsan, & Weimar, 2008). Although it is less expensive than analytical instruments, it presents a lack of sensitivity due to the complexity of the matrix.

Artificial mouths have been presented as an alternative to the conventional SE for the analysis of the retronasal perception (aftertaste). Mastication is simulated by variable-speed motors and artificial saliva is employed, which is usually composed by $NaHCO_3$, NaCl, KCl, $CaCl_2{\cdot}2H_2O$, K_2HPO4, mucin, α-amylase and NaN_3 (in water). The volatile compounds are usually trapped in SPME fibres and desorbed in the GC/MS (Poinot, Arvisenet, Grua-Priol, Fillonneau, & Prost, 2009).

Although less used, other possibilities have emerged in the analysis of food aroma (Cserháti, 2010; Zellner et al., 2012), namely: microwave-assisted hydro distillation (MAHD), pressurized-fluid extraction (PFE), matrix solid-phase dispersion (MSPD) and solid-phase extraction (SPE).

1.2.1.5. Determination of volatile compounds by gas chromatography (GC)

The majority of odours are volatile, with a vapour pressure below 350-400 °C (Cserháti, 2010), which turns GC in the most preferred technique for their separation, determination and quantification. The chromatographic conditions mainly depend on the polarity and boiling point of the studied volatile compounds, which determines the kind of GC column. On non-polar stationary phases compounds are resolved according to their boiling points, while on polar phases compounds are separated in relation to their polarity (Zellner et al., 2012). Undoubtedly, polar stationary phases have been nowadays the most employed in GC food aroma analyses, usually containing 100% polyethylene glycol (Pico, Gómez, et al., 2016).In all the stationary phases, there are lists of retention index reported; most adopted approaches have been Kovats Index (KI) and Linear Retention Index (LRI), which is based on the retention behaviour of the compounds of interest according to a uniform scale determined by a series of alkanes (Zellner et al., 2012). They allow a tentative identification of the volatile compounds, with databases that contain more than 5000 LRI identified (Mottram, 2010).

The first detectors employed were flame-ionisation (FID), nitrogen-phosphorous (NPD), electron capture (ECD) and thermal conductivity (TCD) detectors. However, nowadays almost any food aroma investigation relies on mass spectrometric (MS) detectors, GC/MS. Less frequent in food aroma, but not less important, has been the use of multi-dimensional GC techniques. They are employed with complicated aroma mixtures, when one-dimensional GC is insufficient to separate all the compounds. There are two options: **(i)** Heart-Cut Multidimensional GC (heart-cut MDGC), which selected bands of overlapping compounds are transferred from a primary to a secondary column (Deans, 1968); **(ii)** Comprehensive Two-Dimensional GC (GC×GC), provides an orthogonal two-column separation, with the complete sample transfer achieved by means of a modulator able to continuously trap, refocus, and release fractions of the GC effluent from the first dimension, onto the second dimension column (Liu & Philip, 1991).

Olfactometric detectors, GC/O, have been of utmost importance due to the selectivity and sensitivity of the human nose, enabling the differentiation of multitude of volatile compounds as “odour active” and “non-odour active” compounds, according to their concentrations in the matrix. In GC/O, the effluent of the column is divided into a FID/MS detector and a sniffing port where a human judge smells. Different approaches have allowed the determination of the sensory relevance of an odour, being classified in four categories: dilution, time-intensity, detection frequency, and posterior-intensity methods (Zellner et al., 2012). One of the most important has been Aroma Extract Dilution Analysis (AEDA) for the calculation of the flavour dilution factors (FD). In AEDA, the extract coming from the solvent extraction is diluted, usually as a series of 1:1 or 1:2, and each dilution is analysed again by GC-O until no odour is detected (Belitz et al., 2009). Thus, FD factors are defined as the ratio of the concentration of the volatile in the initial extract to its concentration in the most dilute extract in which the odour is still detectable (Birch, Petersen, & Hansen, 2014). FD factors are a screening method that must be complemented with the calculation of the odour activities values (OAVs), because of the FD factors limitations. OAVs are defined as the ratio of the concentration of the volatile compound in the food product to the compound odour threshold (OT) values in water (Birch et al., 2014); the quantification of the compounds in food could be made using a matched-matrix calibration curve or even Stable Isotope Dilution Assay (SIDA) quantification (Grosch, 1993).

1.2.1.6. Determination of volatile compounds by proton-transfer reaction/mass spectrometry (PTR/MS)

GC/MS presents the drawback of needing long runtimes (Blake, Monks, & Ellis, 2009). Faster techniques are required if it is necessary to monitor a variety of specific volatile compounds on a time scale of 1 min or less. This means that there cannot be a chromatographic separation and, therefore, a soft ionisation is required in order to identify the volatile compounds. PTR-MS, developed in 1960 by Ferguson and co-workers, is a direct injection MS technique based on the application of a pure beam of hydronium ions in a Selected Ion Flow Tube (SIFT) to achieve mild ionisation. Proton transferences take place in order to determine the concentration of the volatile compounds through the kinetic of the reaction. The proton source is normally H_3O^+, although $NH4^+$ could also be utilised (Blake et al., 2009). TOF has normally been the mass analyser employed (**Figure 3**), accomplishing high sensitivities. The main disadvantages are the isobaric interferences, the fragment of ions that match with the same parent ion as well as the isomers. It is suggested that the best option to solve these problems would be the coupling of PTR-MS with fast-GC (Blake et al., 2009), which using adequate columns and instrumentation provides 3- to 10-times faster analysis than conventional GC (Korytár et al., 2002).

1.3. The aroma of bread and related products

1.3.1. Technology of breadmaking

The baking of bread is one of the oldest human activities (Cauvain, 1998). Bread has been defined as a staple food of any size and shape formed by dough made from flour, water and yeast, with or without other ingredients, which has been fermented or otherwise leavened and subsequently baked or partially baked

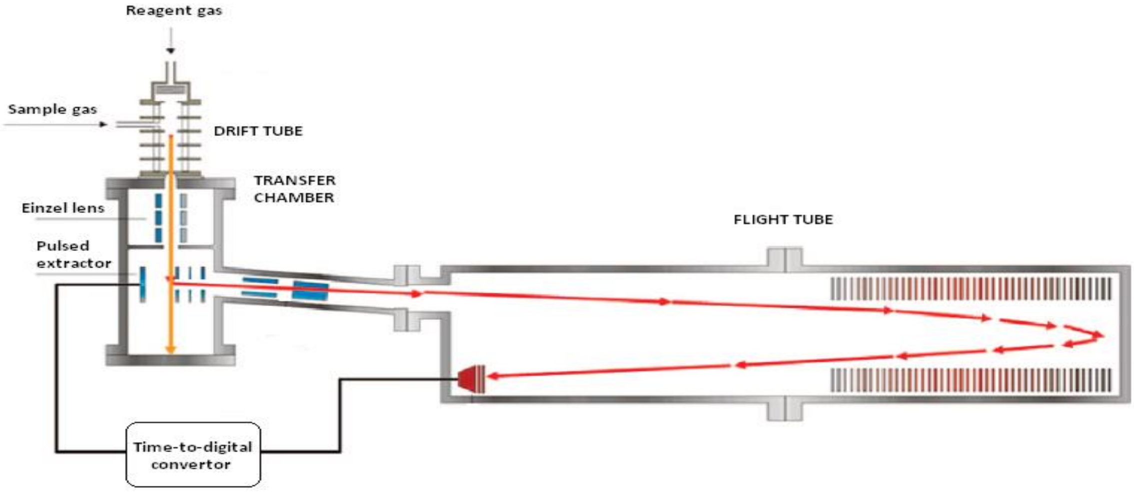

Fig.3. *Scheme of PTR/TOF-MS (from Blake et al., 2009)*

(Edwards, 2007). Nutrients supplied by bread consumption in industrial countries provide close to 50% of the daily requirement of carbohydrates, one third of the proteins, 50-60% of vitamin B as well as minerals, fibres, lipids and trace elements (Belitz et al., 2009). The daily consumption of bread per person advised by World Health Organisation (WHO) in the report EUR/03/5045414 (2003) depended on the country: 250 g for Austria, Armenia and Germany, 290 g for Ukraine, 360 g for Georgia but 25g for Greece and Turkey and 60 g for Spain and Czech Republic.

Bread quality fits with three broad categories (Cauvain, 1998): **(i)** external character, including volume, moisture loss, appearance, colour and crust formation; **(ii)** internal character, namely the size, number and distribution of cells in crumb, crumb colour and any major quality defects, such as unwanted holes; **(iii)** texture, flavour and eating quality, including softness, firmness, cohesiveness, springiness, resiliency and aroma. The choice of the raw materials, the formulation as well as the breadmaking steps, influence the final quality of bread. Actually, there is a relationship between the processing stages and the changes in the composition and structure from the dough to the final bread, as can be seen in **Figure 4**. The kind of flour, the addition of salt, sugar or fat, the dough leaving

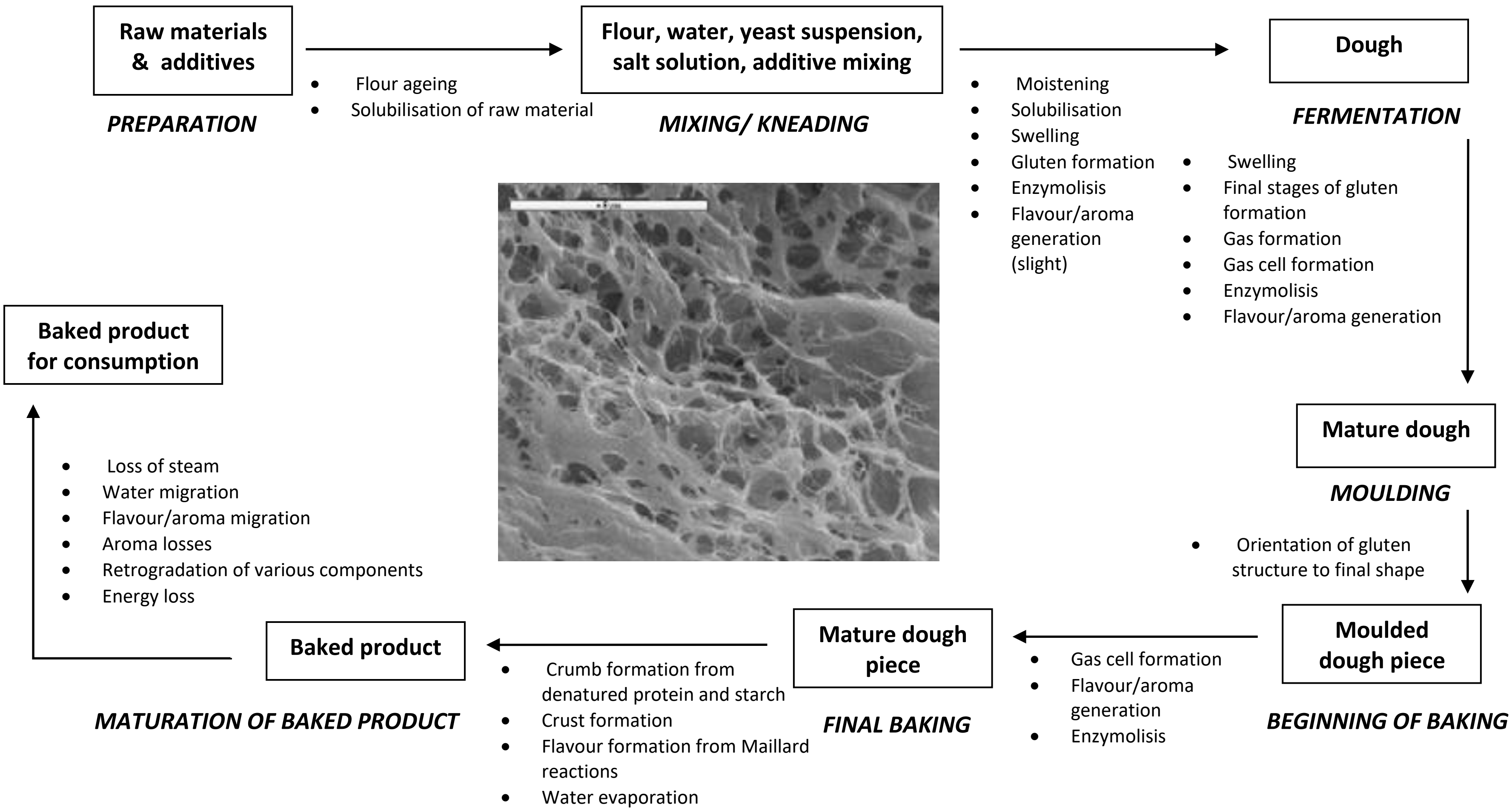

Fig. 4. *Relationship between the processing stages and the changes in the composition and structure from the dough to the final bread (adapted from Stear, 1990)*

agents (yeast or chemical agents), the use of emulsifiers and additives (i.e. ascorbic acid, bromated, or azodicarbonamide) as well as the use of enzymes (e.g., amylases, proteases, lipoxygenases, lipases, etc) will influence the quality properties of bread (Belitz et al., 2009; Cauvain, 2003; Cho & Peterson, 2010; Martínez-Anaya, 1996).

The principal steps in the elaboration of bread are (Cauvain, 2003; Pico et al., 2015): **(i) Kneading**, where the gluten structure starts to be created and the introduction of air creates the dough nuclei bubbles and provides oxygen for yeast and lipoxygenase activities; **(ii) Fermentation**, usually by *Saccharomyces Cerevisiae*, where the starch from the flour is progressively converted into dextrins and sugars by enzyme action. Yeast feeds on the sugars to produce, mainly, carbon dioxide and ethanol. The carbon dioxide fills the air bubbles and causes the dough expansion within its structure. Fermentation usually takes place in a controlled atmosphere between 30°C and 45°C and 85% of relative humidity (RH); **(iii) Baking**, with typical temperatures between 190°C and 250°C, achieving a core temperature of 92-96 °C. There is a heat transfer due to the gradient temperatures from the regions near the crust to the centre. As dough warms up it goes through a progression of physical, chemical and biochemical changes: **(a)** yeast activity decreases from 43°C and ceases by 55°C; **(b)** gelatinization of the starch starts at about 60°C; **(c)** α-amylase activity converts the rest of starch into dextrins and sugars and reaches its maximum activity between 60 and 70°C; **(d)** water is evaporated and the crust acquires its characteristic crispness, colour and flavour (including taste and aroma) developed from the Maillard reactions, which start at temperatures above 110°C.

1.3.2. The aroma of bread from a biological and sensorial point of view

Among the different parameters that define bread quality, flavour is one of the most appreciated sensory attributes (Caul, 1972). In fact, the concept of bread flavour has been the most contentious of all the quality parameters (Cauvain, 2003). More than 540 volatile compounds have been reported in bread, although only a relative small portion of the volatile compounds in bread contribute to the desirable aroma properties (Cho & Peterson, 2010). These compounds are called "aroma compounds" and they can be defined as the volatile compounds that are likely to be sensed when the bread is eaten. Concretely, volatile compounds with OAVs higher than 1 are considered aroma compounds, since their concentration in bread is higher than the corresponding OT. The **Table 1A** (Birch et al., 2014) (see Annex) reflects the most important aroma compounds in wheat bread that have presented OAVs higher than 0.1 and FDs higher than 8. Nevertheless, not only the OAV should be taken into consideration, but also the impact of the volatile compound on the aroma. There are some volatile compounds that have a positive correlation with the final aroma of bread, that is to say, when the concentration of the volatile compound increases, there is also an increase in the pleasant perception of the bread aroma. On the contrary, a negative correlation means that an increase in the concentration of the volatile compound, involves a decrease in this pleasant perception. 3/2-methyl-1-butanol,phenylethyl alcohol, 3/2-methylbutanal, phenylacetaldehyde, furfural, 2,3-butanedione, acetoin, hexyl acetate, 3/2-methylbutanoic acid or 2-acetyl-1-pyrroline have been some of the volatile compounds reported to positively correlate with the final aroma of bread, while 1-octen-3-ol, 2,4-(E,E)-decadienal, benzaldehyde, hexanal, methional or butyric acid have been some of the volatile compounds that have negatively correlated (Pico et al., 2015). Although most of the volatile compounds from fermentation correlate positively, 3-methylpyridine, 4-methylphenol and propanoic acid have been reported as potential off-flavour with high FD generated by yeast (Zhang, Song, Li, Yao, & Xiong, 2017).

Tables 2A to 8A (see Annex) contains 326 volatile compounds reported in the literature commonly identified in wheat bread. The most cited has been 3-methyl-1-butanol, since it has been considered the most important aroma compound from fermentation as a result of its high OVA (Birch et al., 2014), its high FD (Gassenmeier & Schieberle, 1995) and its positive correlation with the final aroma of bread (Rehman, Paterson, & Piggott, 2006), although in really high concentrations it imparts off flavours (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). The second most cited has been hexanal, which is the result of the lipids oxidation (Birch, Petersen, Arneborg, & Hansen, 2013; Jensen, Ostdal, Skibsted, & Thybo, 2011; Quílez, Ruiz, & Romero, 2006). It generates off flavours (Martínez-Anaya, 1996; Quílez et al., 2006), showing high OAV and high concentration in crumb (Arthur & Pawlyszin, 1990; Birch, Petersen, Arneborg, et al., 2013). Finally, the third most studied has been 2,3-butanedione, generated by Strecker degradation (Birch, Petersen, Arneborg, et al., 2013; Birch, Petersen, & Hansen, 2013) and by the Ehrlich pathway (Gassenmeier & Schieberle, 1995). It has presented high OAV (Birch, Petersen, Arneborg, et al., 2013) and high FD (Cho & Peterson, 2010), with pleasant caramel-like notes (Pico et al., 2015). From the heterocyclic compounds, 2-acetyl-1-pyrroline has been has been suggested as a key odorant of the crust generated from Maillard reactions (Zehentbauer & Grosch, 1998a), responsible for the pleasant cracker-like odour properties (Cho & Peterson, 2010) and one of the major active compounds in the crust (Moskowitz et al., 2012) with an OT of 0.053 µg Kg^{-1} (Schieberle & Grosch, 1991).

In general, it can be stated that the aroma of the crumb is mainly generated by fermentation (including the glycolysis of pyruvic acid and the Ehrlich pathway), lipid oxidation processes and reactions in the yeast cell catalised by acetyltransferases. On its part, the aroma of the crust is mainly generated by Maillard reactions (including Strecker degradation), caramelisation and thermal degradation of sugars and amino acids (Pico et al., 2015). However, the transferences of volatile compounds from the crumb to the crust and vice versa during baking are really common (Onishi, Inoue, Araki, Iwabuchi, & Sagara, 2011b). Therefore, it is possible to find 3-methyl-1-butanol in crust as well as traces of pyrazines in crumb. **Figure 5**

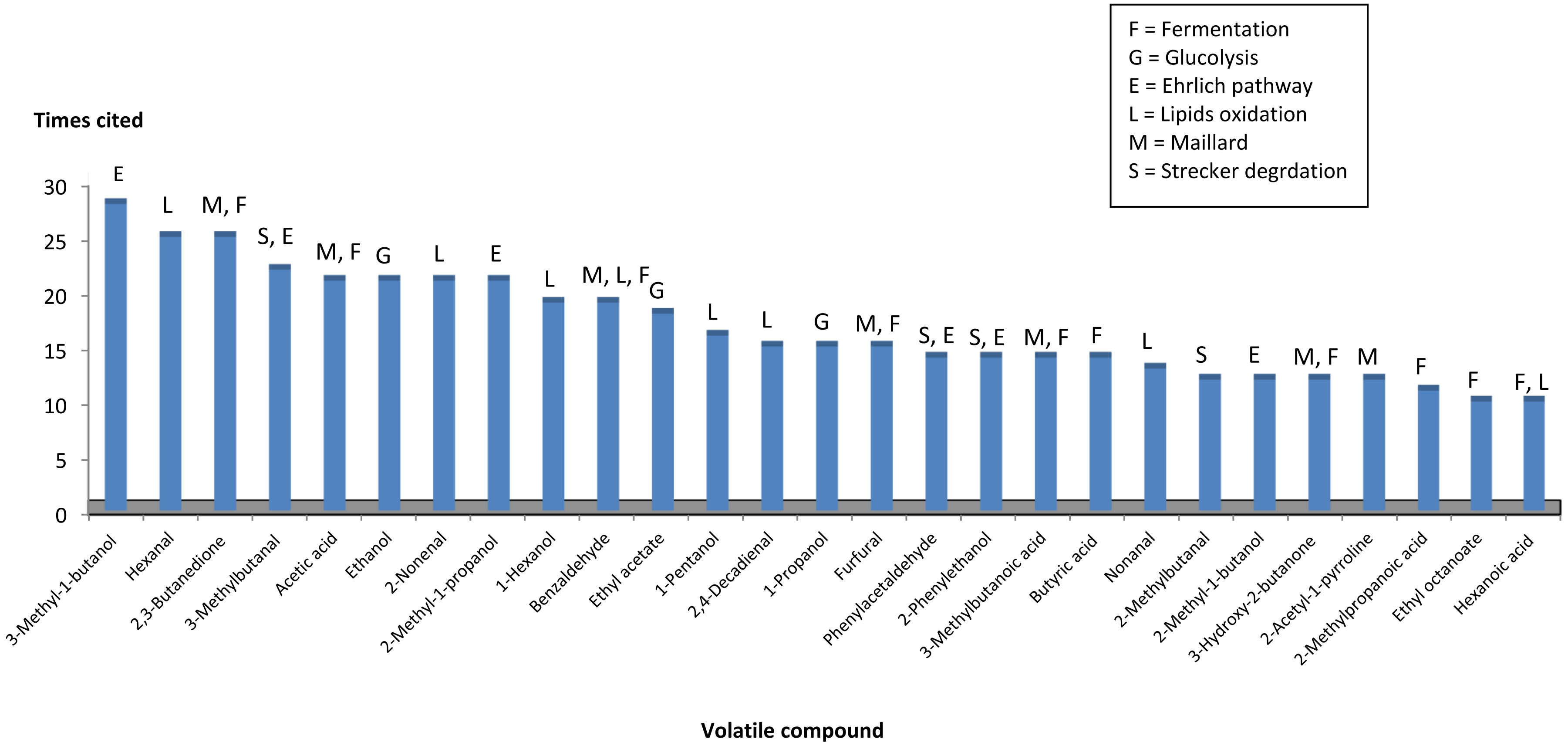

Fig. 5. *M*ost studied volatile compounds in wheat bread crumb and crust according to times cited (at least ten citations) and their biological origin (adapted *from Pico et al., 2015)*

compresses the 28 volatile compounds most cited in the literature classified by their biological origin.

Fermentation produces alcohols, aldehydes, acids, esters and ketones (Bianchi, Careri, Chiavaro, Musci, & Vittadini, 2008; Cho & Peterson, 2010; Martínez-Anaya, 1996) by the action over the sugars of yeasts, above all *Saccharomyces Cerevisiae*, and lactic acid bacteria (LAB), highlighting these of the genus *Lactobacillus* and *Kluyveromyces* (Pico et al., 2015). *Saccharomyces Cerevisiae* is naturally

present in the flour in small concentrations, but it is usually added to the dough in order to promote the fermentation against the lipid oxidation; when there is no addition of yeast, oxygen is more available and it is used by lipoxygenase enzymes to generate aldehydes from the oxidation of lipids usually with off-flavours (Poinot et al., 2008). However, when *S. Cerevisiae* is added, it uses the oxygen in an aerobic process to grow during kneading, encouraging the generation of volatile compounds from fermentation (anaerobic process). In fact, Frasse, Lambert, Richard-Molard, & Chiron (1993) reported that if there was no external addition of S. cerevisiae, the major aroma impact compounds in dough were 2,4-(E,E)-decadienal, 2-(E)-nonenal, from lipid oxidation, and methional, from Ehrlich pathway. However, when S. cerevisiae was added to the dough, the major aroma impact compounds were 3-methyl-1-butanol, 2-methyl-1-butanol, 2,3-butanedione, methional and 2-phenylethanol, all of them from fermentation.

95% of these sugars are fermented by *S.Cerevisiae* into ethanol and carbon dioxide, while the remaining 5% participate in secondary fermentation reactions, namely *glycolisis of pyruvic acid* (Czerny & Schieberle, 2002) and the *Ehrlich pathway* (Birch, Petersen, Arneborg, et al., 2013), both processes being summarised in **Figure 6**. Short chain alcohols, short chain fatty acids, esters and carbonyl compounds are generated during the glycolisis of pyruvic acid (Drapon & Richard-Molar, 1979), while higher molecular weight alcohols are obtained from the Ehrlich pathway through the transamination, decarboxilation and reduction/ oxidation of

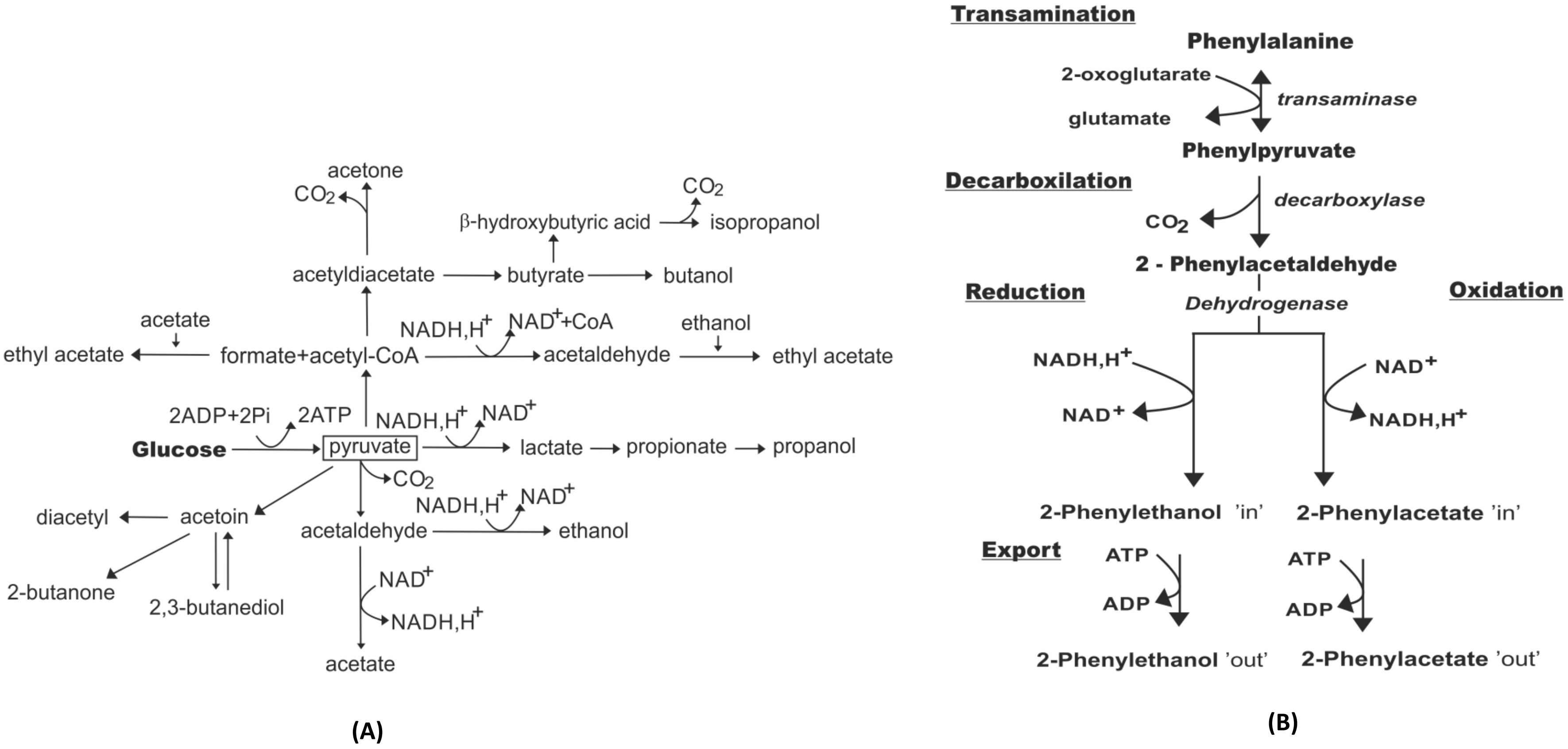

Fig. 6.*Volatile compounds generated during fermentation by glycolysis of pyruvic acid (A) and by Ehrlich pathway of phenylalanine (B) (adapted from Pico et al. 2015)*

initial amino acids. In this way, **(i)** 3-methylbutanal, 3-methyl-1-butanol and 3-methylbutanoate derive from leucine; **(ii)** 2-methylpropanal, 2-methylpropanol and 2-methylpropanoate from valine; **(iii)** 2-methylbutanal, 2-methyl-1-butanol and 2-methylbutanoate from isoleucine; **(iv)** 2-phenylethanal, 2-phenylethanol and 2-phenylethanoate from phenylalanine; **(v)** methional, methionol and 3-(methylthio)-propanoate from methionine (Hazelwood et al., 2008).Finally, regarding the action of LABs, they are normally added to the recipe using sourdough (Plessas, Fisher, et al., 2008; Ur-Rehman et al., 2006). The coexistence of yeast and LABs generates a greater number of volatile compounds than if only LABs were added (B. Hansen & Hansen, 1994). However, if the dough already contained yeasts, LABs do not significantly modify the volatile profile, just an increase in the short-chain organic acids (Martínez-Anaya, 1996). Naturally, the fermentation time and temperature would also affect the generation of volatile compounds (Cauvain, 1998; Wiggins, 1998; Zehentbauer & Grosch, 1998b).

Lipid oxidation includes the generation of aldehydes, alcohols, ketones and furans (Birch et al., 2014), through the transformation of polyunsaturated fatty acids (mainly linoleic and linolenic acids) in hydroperoxides, which are unstable and degrade during baking into a great number of volatile compounds (Pico et al., 2015), as it is shown in **Figure 7**. Lipoxygenases are the enzymes responsible of using the oxygen to transform the unsaturated fatty acids into volatile compounds, namely 2,4-(E,E)-decadienal, nonanal, 2-(E)-nonenal, octanal, heptanal, 1-pentanol, 1-hexanol, 1-octen-3-one or 2-pentylfuran (Birch et al., 2014; Pico et al., 2015). Therefore, the availability of the oxygen as well as the amount of polyunsaturated fatty acids in the flour, have been considered key factors. Moreover, the lipoxygenase activity differs between different flours, since rice and wheat present high lipoxygenase activities (Leenhardt et al., 2006; Wongdechsarekul & Kongkiattikajorn, 2010), while in quinoa flour it is almost negligible (Caussette, Kershaw, & Shelton, 1997). Finally, the content of antioxidants, such as vitamin E and flavonoids, is also important in order to inhibit the lipoxygenase action (Pico, Hansen, & Petersen, 2017).

Maillard reactions refer to the chemical reactions between an amino acid and a reducing sugar, such as glucose and fructose, in the presence of temperatures between 110°C - 150°C (Onishi, Inoue, Araki, Iwabuchi, & Sagara, 2011a), leading to the formation of brown pigments (melanoidines) and a large number of volatile compounds (Pico et al., 2015), as can be noted in **Figure 8**. Thereby, Maillard reactions generate, principally, furans, pyrazines, pyrroles, pyrrolines, oxazoles, thiophenes, thiopyranes, thiazolines and sulphuric compounds in crust during baking (Bianchi et al., 2008; Bredie, Mottram, & Guy, 2002; Cho & Peterson, 2010). The Strecker degradation is one of the main steps of Maillard reactions and it implies the reaction between amino acids and dehydroreductones to produce

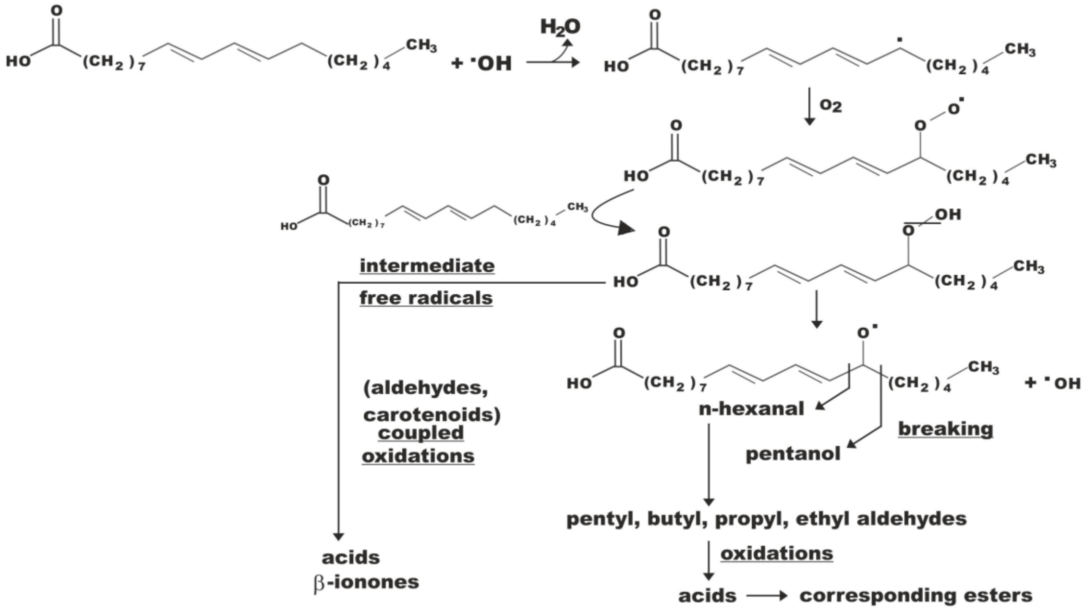

Fig. 7. *Volatile compounds generated through the lipid oxidation of linoleic acid (adapted from Pico et al. 2015)*

aldehydes with the same structure of the former amino acid (Guinet & Godon, 1996). Consequently, acetaldehyde comes from alanine, formaldehyde from glycine, glyoxal from serine, 2-hydroxypropanal from threonine (Rooney, Salem, & Johnson, 1967) and, also generated through the Ehrlich pathway, 2-methylpropanal from valine, 3-methylbutanal from leucine, 2-methylbutanal from isoleucine, phenylacetaldehyde from phenylalanine and methional from methionine (Martínez-Anaya, 1996; Rooney et al., 1967).

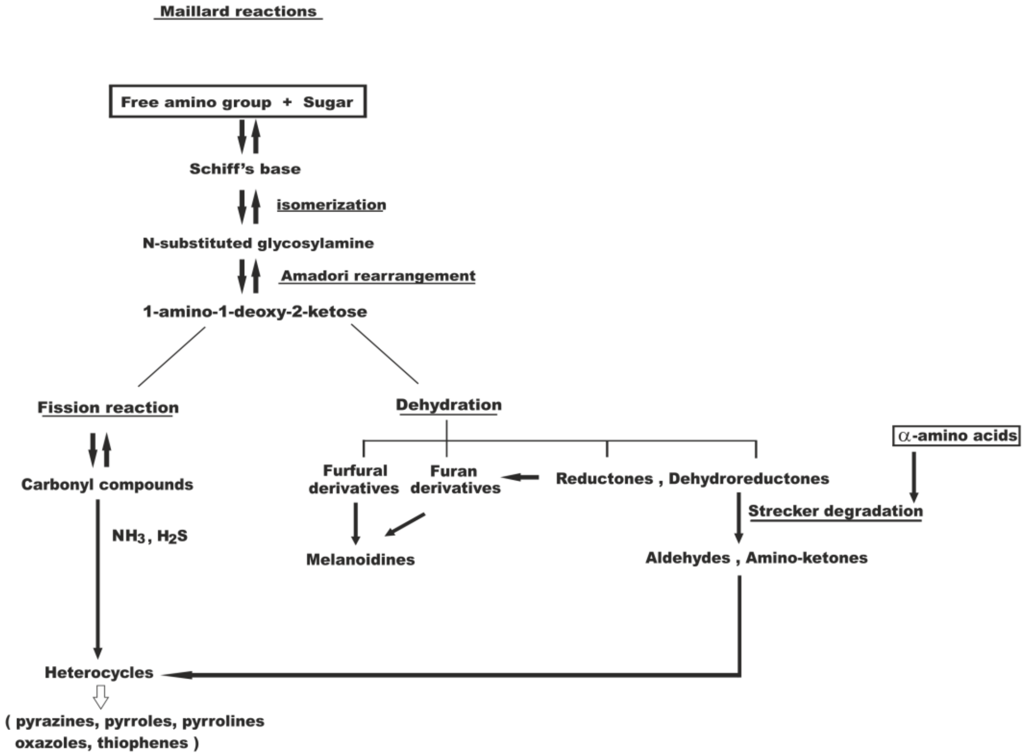

Fig. 8. *Volatile compounds produced by means of Maillard reactions (adapted from Pico et al. 2015)*

The rate of the Maillard reaction depends on the type of sugar, being xylose the most reactive, whilst the aroma profile relies on the type of amino acid (Kiely, Nowlin, & Moriarty, 1960). On the other hand, the temperature acts as a catalyst and the humidity of the oven favours the starch dextrinisation and generation of sugars for Maillard reaction (Guinet & Godon, 1996). 2-acetyl-1-pyrroline has been considered the key aroma compound of Maillard reaction, but 2-methylbutanal, 3-methylbutanal, methional (all three are also generated by the Ehrlich pathway), 3-methylbutyric acid, 2,3-butanedione (also from fermentation), 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 2-methyl-propanal, were also important Maillard compounds due to their high OAV. Special mention should be made to acrylamide, furan and 5-hydroxymethylfurfural, since they are known as potentially harmful compounds associated to mutagenic, carcinogenic and cytotoxic effects (Capuano & Fogliano, 2011). Although acrylamide is not very volatile (boiling point 241 °C), it is commonly present in bread, generated from asparagines degradation with reducing sugars (Mottram, Wedzicha & Dodson, 2002). 5-hydroxymethylfurfural can be generated by Maillard reactions and caramelisation, while furan is generated by lipid oxidation, thermal degradation of ascorbic acid and Maillard reactions (Rannou, Laroque, Renault, Prost, & Sérot, 2016).

Caramelisation is also a thermal reaction that takes place at temperatures higher than 150°C (Hadiyanto et al., 2007; Onishi et al., 2011a), when the sugars are heating above their melting point. The range of temperatures can be overlapped with the Maillard reaction and some volatile compounds can be generated by both sources, specially furan derivatives, such as furfural, 5-methyl-2-furaldehyde and furfuryl alcohol (Ait Ameur, Rega, Giampaoli, Trystram, & Birlouez-Aragon, 2008). Furfural is generated by the reaction between phenylalanine and xylose (Nakama, Kim, Shinohara, & Omura, 2014) and 5-methyl-2-furaldehyde is derived from phenylalanine and rhamnose (Buera, Chirife, Resnik, & Wetzler, 1987). However, both furfural and 5-methyl-2-furaldehyde could be produced by pentose caramelisation (Ait Ameur et al., 2008). Furfuryl alcohol is the only mainly reported as a reduction product from furfural (Spillman, Pollnitz, Liacopoulos, Pardon, & Sefton, 1998).

1.3.3. Analysis of volatile compounds in bread

Some studies have analysed the whole bread, which was frozen with liquid nitrogen and ground into a powder (Jensen et al., 2011; Keshri, Voysey, & Magan, 2002; Lin et al., 2009; Luning, Roozen, Moëst, & Posthumus, 1991; Paraskevopoulou, Chrysanthou, & Koutidou, 2012; Plessas, Fisher, et al., 2008; Plessas et al., 2011; Plessas, Bekatorou, et al., 2008; Poinot et al., 2007, 2008, 2010). However, the most common is the separation of the crumb and crust, since it is possible to obtain clearer information. There are some studies that have been focused on the crumb (Birch, Petersen, Arneborg, et al., 2013; Birch, Petersen, & Hansen, 2013; Gassenmeier & Schieberle, 1995; Hansen & Hansen, 1996; Kirchhoff & Schieberle, 2001; Maeda et al., 2009; Ruiz et al., 2003; Rychlik & Grosch, 1996), other studies on the crust (Moskowitz et al., 2012; Pacyński, Wojtasiak, & Mildner-Szkudlarz, 2015; Schieberle & Grosch, 1985, 1994) and some of them that have analysed both the crumb and crumb (Onishi et al., 2011b; Schieberle & Grosch, 1991; Zehentbauer & Grosch, 1998a). If necessary, the powder has been spiked with an internal standard (IS) prior to the analysis. Onishi et al. (2011b) employed 3-heptanol as IS for aroma analyses of both crumb and crust by solvent extraction. 2-methyl-3-heptanone has been also reported as an IS for aroma analyses of crust by solvent extraction (Moskowitz et al., 2012) and 4-methyl-2-pentanol and 2-ethylbutyric acid as IS for crumb SPME analysis. Antioxidants, like butylated hydroxytoluene (BHT), have been also utilised in spiked samples in order to prevent oxidation (Moskowitz et al., 2012).

Regarding solvent extraction, most of them have employed Soxhlet extraction (Gassenmeier & Schieberle, 1995; Kirchhoff & Schieberle, 2001; Onishi et al., 2011b; Pico, Martínez, Bernal, & Gómez, 2017a, 2017b; Pico, Nozal, Gómez, & Bernal, 2016; Rychlik & Grosch, 1996; Zehentbauer & Grosch, 1998a), and only in a few cases overnight classical agitation extraction at room temperature has been carried out (Moskowitz et al., 2012). All of them have used dichloromethane and only in a few cases diethyl ether was employed (Zehentbauer & Grosch, 1998a). For the isolation of the volatile compounds from the non-volatile matrix, VS was the most common in the past (Gassenmeier & Schieberle, 1995; Rychlik & Grosch, 1996; Schieberle & Grosch, 1985, 1991, 1994; Zehentbauer & Grosch, 1998a), but SAFE is the most usual nowadays (Moskowitz et al., 2012; Onishi et al., 2011b). SDE has not been usually utilised (Lin et al., 2009), probably due to the problems of artifact generation. All of them have employed GC/MS, and less frequent GC/FID, for the determination and quantification of the volatile compounds. Lastly, the use of the GC/O has been really important in order to determine which compounds were really contributing to bread aroma, using the FDs and OAVs (see sub-section 1.3.2).

Concerning the headspace methodologies, SHS has been the less employed (Maeda et al., 2009) due to the lack of sensitivity. DHS has been sometimes employed (Birch, Petersen, Arneborg, et al., 2013; Birch, Petersen, & Hansen, 2013), but without any doubt, SPME has been the preferred technique (Pacyński, Wojtasiak, & Mildner-Szkudlarz, 2015b; Pauline Poinot et al., 2007, 2010; Ruiz et al., 2003), most likely due to small amount of sample required and the great number of possibilities choosing the selectivity of the fibre. The DHS and SPME conditions usually used in the analysis of bread aroma are collected in **Table 3** and **Table 4**, respectively.

Finally, in relation to other options, such as SFE and electronic nose or mouth, their use has not been spread. In fact, there is no report of the use of SFE in the analysis of bread aroma. The usefulness of the electronic nose is limited to the quality control of determined breads (Botre & Gharpure, 2006; Sapirstein, Siddhu & Aliani, 2012) in order to observe changes in the total amount of gas comprised of volatile compounds, without considering the type of volatile compounds present (Pico, Gómez, et al., 2016). Finally, the use of electronic mouth for the retronasal volatile compounds in bread has been more extended since the volatile compounds are first extracted with artificial saliva but then they are absorbed in SPME fibres and

Table 3. DHS conditions for bread aroma commonly reported in the literature

Bread weight	Trap	Inert gas	Extraction T/time	Desorption T/time	Carrier gas	GC detector	References
15 g	Tenax-TA	N_2 (150 mL/min)	40°C/60min	250°C/15min	H_2	MS	Birch et al., 2013a
25 g	Tenax-TA	N_2 (50 mL/min)	26°C/30min	300°C/3min	H_2	MS	Jensen et al., 2011
90 g	Tenax-TA	N_2 (20 mL/min)	Room T/7h	180°C/10min	H_2	MS & FID	Luning et al., 1991
30 g	Tenax-TA	N_2 (40 mL/min)	60°C/8min	200°C/4min	H_2	MS	Seitz et al., 1999
1.5 g	Tenax-TA	H_2 (40 mL/min)	40°C/15min	280°C/10min	H_2	MS	Bianchi et al., 2008

Table 4. SPME conditions for bread aroma commonly reported in the literature

Bread weight	Fibre	Salt	Extraction T/time	Desorption T/time	GC detector	References
0.25 g	CAR/PDMS	20% NaCl (pH 3)	50°C/60min	300°C/5min	MS	Ruiz et al., 2003
6 g	CAR/PDMS & CAR/PDMS/DVB	Not used	35°C/30min	260°C/5min	MS&O	Poinot et al., 2007
6 g	CAR/PDMS	Not used	35°C/35min	260°C/5min	MS	Poinot et al., 2008
2 g	CAR/PDMS/DVB	Not used	60°C/60min	280°C/5min	MS	Plessas et al., 2011
3 g	CAR/PDMS/DVB	20% NaCl (pH 3)	60°C/60min	250°C/4min	MS & O	Paraskevopoulou et al., 2012
2 g	CAR/PDMS/DVB	Not used	60°C/60min	280°C/5min	MS	Plessas et al., 2008

desorbed in the GC/MS (Onishi, Inoue, Araki, Iwabuchi, & Sagara, 2012; Poinot et al., 2009). In the same way, the use of PTR/MS for the analysis of bread aroma has not been extended yet, probably due to the challenges with the isobaric interferences. Its use has been focused on the on-line monitoring of bread due to the high throughput, such as the fermentation and baking of bread (Capozzi et al., 2016; Makhoul et al., 2014) or the release of volatile compounds during bread mastication (Jourdren et al., 2017a; Jourdren et al., 2017b; Onishi et al., 2009).

1.3.4. Improving the aroma of bread

The analysis of volatile compounds in bread using analytical techniques can be applied to the routine quality control. However, the main usefulness of the conclusions extracted from the bread volatile profile is found in the improvement of its aroma. This is even more useful in the case of gluten-free breads (see sub-section 1.4), since they have been characterised by a sensory quality barely acceptable, almost notably the texture and aroma (Pacyński et al., 2015). Until now, there have emerged several options for the improvement of bread aroma:

(i) Breadmaking stages. The increase in the kneading time has led to losses of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal (Zehentbauer & Grosch, 1998b). These Strecker and Ehrlich aldehydes have been characterised for a pleasant malty odour related to the freshness of bread (Pico et al., 2015; Zehentbauer & Grosch, 2010). Moreover, isovaleric and butyric acids, which show unpleasant notes (Pico et al., 2015), have been also associated to high-intensity kneading processes (Quílez et al., 2006). As a consequence, short kneading times would be required for the improvement of the aroma of bread. Regarding the fermentation step, it can be said that, in general, more aromatic breads need longer fermentation times at low temperatures (Birch et al., 2013; Pico et al., 2015) for achieving a great amount of pleasant volatile compounds from fermentation.

(ii) Type of flour. Undoubtedly, the kind of flour influence the final aroma of bread due to their precursors of fermentation and caramelisation (small amount of free sugars and starch), lipids oxidation (polyunsaturated fatty acids) and Maillard reactions (free amino acids and proteins). Moreover, it comprises small amounts of endogenous enzymes, including lipoxygenases for the lipids oxidation. The antioxidants present in flour also influence the lipids oxidation reactions. Finally, it also encloses small amounts of microorganisms, mainly yeasts and LABs, which can participate in the fermentation process and also provide additional enzymes from their metabolic activity. The participation of volatile compounds from the flour in the final aroma of wheat bread has been found to be of minor importance (Cho & Peterson, 2010).

(iii) The use of sourdough. There have been a great number of studies focused on the aroma of sourdough breads in the last decades (Hansen & Hansen, 1994; Hansen & Hansen, 1996; Hansen & Schieberle, 2005; Kirchhoff & Schieberle, 2001; Plessas et al., 2011; Plessas, Bekatorou, et al., 2008; Ur-Rehman et al., 2006; Vermeulen, Czerny, Gänzle, Schieberle, & Vogel, 2007; Wolter, Hager, Zannini, Czerny, & Arendt, 2014). Sourdough is a mixture of flour and water that is fermented using yeasts and LABs, employed for dough leavening but, above all, employed to extend the shelf life, nutritional properties and bioactive compounds in bread. However, today it is mostly used as a bread flavour improver (Pétel, Onno, & Prost, 2017).

(iv) Addition of improvers and nutrients. The current trend of improving the nutritional properties of bread should be complemented by a good flavour.

(a) Inulin, a fibre added to prevent intestinal diseases (Franck, 2008), has been proved to accelerate the formation of the crust and the Maillard reactions (Poinot et al.,2008; Poinot et al., 2010). However, the bread loaves are smaller with a harder crumb, consequently fortifications higher than 5% should not be used (Morris & Morris, 2012); (b) Fructo-oligosaccharides (FOS) are also prebiotics that also impart health benefits. As inulin, it also showed a speed up in the formation of the crust and in the Maillard reactions (Morris & Morris, 2012); (c) Chestnut flour has been employed to supplement breads for its antioxidant and nutritional properties (De Vasconcelos, Bennet, Rosa, & Ferreira-Cardoso, 2010). When wheat bread is supplemented with chestnut flour (20%), the obtained volatile profile is richer, with a marked increase of furans, with their toasty and nutty notes, and phenolic compounds, with their woody and smoky notes (Dall'Asta et al., 2013).

(v) Addition of enzymes. They have been commonly employed as bread improvers (Belitz et al., 2009). Amylases and glucosidases are responsible for the production of reducing sugars, which then act as fermentation substrates and also participate in Maillard reactions and caramelisation (Guinet & Godon, 1996). Proteases produce peptides and amino acids that are metabolised by yeasts during the Ehrlich pathway and could participate in Maillard reactions, including Strecker degradation (Guinet & Godon, 1996). Lipoxygenases produce, through lipid oxidation reactions, unstable products that decompose to off-flavour carbonyl compounds (Martínez-Anaya, 1996). Lipases are also important because they hydrolyse triacylglycerides in 1 and 3 bonds, releasing fatty acids (Guinet & Godon, 1996), which can be utilised by lipoxygenases. These enzymes could act in either a single or a synergistic way.

1.4. The aroma of gluten-free breads

1.4.1. Gluten-related disorders: causes, symptoms and diagnosis

Around 1% of the worldwide population suffer gluten-related disorders, which include wheat allergy, autoimmune disorder and non-celiac sensitivity. The increase of the number of celiac people should have been caused by environmental and genetic factors (Gallagher, 2009), but also by the better diagnostic methods. Beside the gluten-related disorders, there is a new segment of consumers that follow a gluten-free diet just as a lifestyle choice (Foschia, Horstmann, Arendt, & Zannini, 2016). Food can be labelled as gluten-free when there is a complete absence of gluten (Commission Regulation EC No 41/2009) or when the gluten level is under 200 parts per million (ppm) (Codex Alimentarius, 2008).

Wheat allergy is an autoimmune reaction to any of the wheat proteins, not only gluten (although 80% of them are gluten). The presence of wheat proteins provokes the release of the antibodies immunoglobulin E (IgE) from the B lymphocytes (Battais et al., 2006), which can lead to an anaphylactic shock and even the death. This disorder is assessed by an allergist, normally through radioallergosorbent test (RAST) or skin prick test (SPT). Unfortunately, the only current available treatment is a strictly gluten-free diet.

Depending on the symptoms, autoimmune disorders can be divided into celiac disease, ataxia (lack of coordination of the body movements) and dermatitis herpetiforme (itchy skin affection of inflammatory nature). Celiac disease, also called gluten enteropathy or celiac sprue, is one of the most common food diseases in humans (Foschia et al., 2016). It is an immune-mediated enteropathy caused by the ingestion of gluten from cereals of *Triticeae* tribu (i.e. wheat, rye and barley) in genetically susceptible individuals, leading to damages of the small intestinal mucosa (Gallagher, 2009; Naqash et al., 2017). Gluten is composed by 90% of the glutenins and gliadins (proteins type prolamin), 8% of lipids and 2% of carbohydrates. Concretely, gliadin is the toxic fraction for celiac people since it is water soluble, causing bowel permeability, regardless the genetic predisposition (Rodrigo, Garrote & Vivas, 2008). It is the responsible of the extensibility of bread dough. Glutenin is non-toxic for celiac people and it is the responsible of the elasticity of bread dough. Celiac disease is diagnosed by a gastroenterologist through serology test as well as biopsy and histology; biopsy is the most common diagnostic method. It is presented both in children and adults, most common in female, and the usual symptom is diarrhea in the 85% of the cases. Thus, many patients with celiac disease have had a previous diagnosis of irritable bowel syndrome (Gallagher, 2009). As for wheat allergy, the only current available treatment is a strictly gluten-free diet.

Non-celiac gluten sensitivity (NCGS) is not well defined, but it can be understood as a clinical entity induced by the ingestion of gluten leading to intestinal symptoms (Ludvigsson et al., 2013). It is not a reaction of the IgE, like wheat allergy, neither an immune-mediated enteropathy, like celiac disease. Therefore, there are not biomarkers for the identification of NCGS and it cannot be identified by serology or histology tests, biopsy or RAST/SPT. Its diagnosis is made by the exclusion of wheat allergy and celiac disease and it presents the highest incidence among the gluten-related disorders, with prevalence between 0.5-13 % of the general population (Molina-Infante et al., 2015). Again, the only current available treatment is a strictly gluten-free diet.

1.4.2. The importance of gluten and gluten-free breads

Wheat flour is one of the most employed in the making of bread and it is due to the special properties of wheat proteins (Cauvain, 2003). Specifically, gluten is considered an essential structure-building protein, contributing to the appearance, crumb structure, and consumer acceptance of wheat bread (Arendt, Morrissey, Moore, & Dal Bello, 2008). It is the responsible of the dough viscoelasticity as well as the gas retention, mixing tolerance and resistance to stretch (Foschia et al., 2016). Indeed, the solid matrix of the crumb can be considered as a continuous phase of gelatinised starch and gluten network with the starch granules and fibres confined (Durrenberger et al., 2001). The removal of gluten from bread lead to deficiencies of the quality attributes nutritional characteristics and consumer acceptance. Hence, gluten-free breads have been characterised by a deficient gas retention, low volume, poor colour, post-baking quality defects (Naqash et al., 2017), as well as less cohesive and elastic system with a crumbling texture (Foschia et al., 2016). Regarding the nutritional shortages, they present a poor supply of fibres, minerals, vitamins and proteins but a high carbohydrate and fat content (Naqash et al., 2017). As a consequence, gluten-free breads have been described by customers as a “blend of chemically-based food ingredients with unpalatable, frequent artificial flavours” (Rosell & Matos, 2015). Thus, the biggest challenge for food scientists of gluten-free products is probably the production of high-quality gluten-free bread (Arendt, Morrissey, Moore, & Dal Bello, 2008).

Gluten-free breads are a combination of gluten-free flours from cereals (e.g. rice, corn, teff, oat, millet, sorghum), pseudocereals (e.g. quinoa, amaranth, buckwheat), starches (e.g. corn, wheat), tubers (potato, cassava), legumes (soy, chickpea, pea) or nuts (chestnut) with yeast, water, salt but also hydrocolloids, gums, specific enzymes, sugar, proteins, fibres, emulsifiers and fat. It is common and advisable the mixture of more than one type of flour or starch (Arendt, Morrissey, Moore, & Dal Bello, 2008; Foschia et al., 2016). Hydrocolloids, also called gums, are an essential ingredient because they are able to mimic the viscoelastic properties of gluten, yielding bigger gas cells and a reduction in the losses of water. In addition, they can limit the interactions between the starch and proteins, giving softer crumbs and reducing the staling. The most common option has been the use of polymers chemically synthesised from cellulose, like methyl cellulose (MC), carboxymethyl cellulose (CM) and hydroxypropylmethyl cellulose (HPMC) (Foschia et al., 2016; Houben, Höchstötter, & Becker, 2012). The polymers xanthan gum, agar-agar, carrageen, β-glucan, gum arabic, guar gum and psyllium have been alternatives from natural origins (marine, plants, seed mucilage) (Houben et al., 2012).

Peer-reviewed literature on gluten-free bread making has primarily focused on overall efforts to study and improve quality parameters such as nutritional values, rheology of the dough, texture, volume, colour or staling (Alvarez-Jubete, Arendt, & Gallagher, 2010; Houben, Höchstötter, & Becker, 2012; Mancebo, Merino, Martínez, & Gómez, 2015; Masure, Fierens, & Delcour, 2016; Pruska-Kędzior et al., 2008). In fact, there have emerged several approaches for the improvement of gluten-free breads (Foschia et al., 2016; Houben et al., 2012; Naqash et al., 2017). Masure et al. (2016) reviewed the most common recipes studied in gluten-free breadmaking of 132 articles published between 2010 and 2015. The analyses performed on the dough have included rheology, rheofermentometry, rapid visco analyser (RVA), differential scanning calorimeter (DSC) and scanning electron microscope (SEM), while the analyses performed on the bread (crumb and crust) have included texture, DSC, colour, sensory analyses, image and SEM.

1.4.2.1. Analysis of volatile compounds in gluten-free breads: state of art

Although 49 of the 132 articles reviewed by Masure et al. (2016) have accomplished sensory analyses, there is no information about which volatile compounds were the responsible of the preference for particular gluten-free bread. For this purpose, it is necessary to analyse the volatile profiles of gluten-free breads through analytical techniques (see sub-section 1.2.1). Accordingly, there has been until now little knowledge regarding the analysis of volatile compounds in gluten-free breads. Research in gluten-free bread aroma has focused on understanding the origin of the volatile compounds compared to wheat bread (Poinot et al., 2009), the improvement of gluten-free bread aroma based on the method of baking (Aguilar et al., 2015) or the improvement of the crust aroma with the addition of sugar-amino acid pairs to encourage the Maillard reaction (Pacyński et al., 2015). Poinot et al. (2009) reasoned that gluten-free breads were characterised, compared to wheat bread, by higher quantity of volatile compounds from lipids oxidation and lower amount of volatile compounds from fermentation and Maillard reactions; however, they just showed the Principal Component Analysis (PCA) without specifying the studied volatile compounds. Aguilar et al. (2015) found a new technology for baking the gluten-free breads in a microwave using a special packing material (SPM) that gives the same volatile profile that convection oven, leading to a reduction of time and energy. The most abundant compounds were from fermentation, followed by volatile compounds from lipids oxidation. Pacyński et al. (2015) concluded that the main differences between gluten-free and gluten breads were in the volatile compounds of the crust, since gluten-free breads were characterised by a lack of volatile compounds from Maillard reactions. The addition of the pair proline-glucose increased their concentration.

Nevertheless, the three researches employed a commercial preparation for the making of bread. Thus, up to now, there has been no knowledge regarding the influence of the ingredients as well as the breadmaking steps on the final aroma of gluten-free bread. Only with this information it would be possible to develop new recipes for the improvement of the final aroma of gluten-free bread.

II. Justification and objectives

2. Justification and objectives

2.1. Justification

Bread is one of the most consumed staple foods around the world. Among the different properties that define its quality, the aroma, both orthonasal and retronasal, is one of the main characteristics for its acceptance by customers. In this context, the overall quality of gluten-free breads has been described as really poor, above all its flavour, including both aroma and taste. The generation of their volatile compounds is influenced, mainly, by the ingredients and the breadmaking steps. One of the main ingredients is the gluten-free flour or starch, which contains the precursors (i.e. sugars, proteins and lipids) for the reactions that produce a wide range of volatile compounds, as well as the corresponding enzymes, yeasts, LABs and antioxidants. Therefore, the selection of the suitable flour or starch could contribute to the improvement of the aroma of the gluten-free bread. Once the influence of each flour or starch in the final aroma of gluten-free bread has been examined, it would be possible to coherently suggest the most appropriate mixture of flours and starches that lead to gluten-free breads with an improved aroma but also with acceptable physical properties.

Special gluten-free flours have been pseudocereals flours, above all quinoa and amaranth, which have been reported to contain high nutritional values in terms of proteins, lipids, carbohydrates, vitamins, minerals and fibers (Alvarez-Jubete et al., 2010; Hirose, Fujita, Ishii, & Ueno, 2010; Jancurová, Minarovičová, & Dandár, 2009). In comparison to wheat flour, the higher content of proteins in quinoa and amaranth (Alvarez-Jubete et al., 2010) is expected to affect the development of Maillard reactions, Strecker degradation and Ehrlich pathway. Although the content of starch of pseudocereal flours is similar to that of wheat flour, quinoa has been reported to contain greater amount of α-glucosidase (Elgeti et al., 2014) leading to free sugars, which undoubtedly would influence the rate of the fermentation as well as the caramelisation and Maillard reactions. Finally, pseudocereals have been characterised by higher contents of lipids (Alvarez-Jubete et al., 2010), which influence the lipid oxidation reactions. However, not only the level of lipids should be taken into consideration, but also the lipoxygenase and the antioxidant activities. The lipoxygenase activity has been considered to be significant in rice (Wongdechsarekul & Kongkiattikajorn, 2010), while in quinoa it has been considered to be negligible (Caussette et al., 1997); the antioxidant activity in pseudocereals, especially in quinoa, has been described to be higher than in cereals (Laus, Gagliardi, Soccio, Flagella, & Pastore, 2012). Nevertheless, one of the main disadvantages of using gluten-free cereal or pseudocereal flours is the little volume of the corresponding bread. Thus, the inclusion of starches has been important in order to increase the bread volume when using mixtures of starches and gluten-free flours (Mancebo et al., 2015; Mariotti et al., 2013, Onyango et al., 2011). The increase in the volume of bread would affect the perception of its aroma and the use of starches itself would also influence the generation of volatile compounds. Therefore, the importance in the selection of the gluten-free flours and starches has been proved. In the present Doctoral Thesis, cereals flours (white corn, yellow corn, japonica rice, basmati rice, oat and teff), pseudocereal flours (quinoa, amaranth and buckwheat) and starches (corn, wheat and potato) were employed. Wheat flour was selected as the control sample.

2.2. Objectives

The main objective of this Doctoral Thesis has been the improvement of the aroma of gluten-free breads through the study of the influence of different gluten-free flours and starches on the volatile profile of the corresponding bread. For this purpose, different analytical methods were developed, optimised and validated, including solvent extraction and headspace methodologies, both with GC/MS for the determination and quantification of the volatile compounds. The achievement of this aim would allow the elaboration of recipes containing a wide range of mixtures of flours and starches, depending on the requirements of the customers.

The selected mixture should fulfil with acceptable physicochemical parameters, such as texture, volume, colour or crispiness.

In order to achieve the main objective, several specific objectives were also established:

1- Development and validation of an alternative solvent extraction methodology for the quantification of volatile compounds in bread crumb. Comparison of its analytical characteristics with those of the classical SAFE methodology.

2- Examination of the optimal freezing time that preserves the volatile profile of breads at the moment of being analysed.

3- Study of the inhibition of the residual fermentation for the accurate volatile compounds analyses in doughs at different fermentation times.

4- Analysis of the evolution of the volatile profiles at different fermentation times as well as from fermentation (dough) to baking (crumb) in gluten-free breads.

5- Screening of the impact of different gluten-free flours and starches on the volatile profile of the corresponding doughs and crumbs using SHS-GC/MS and examination of the influence of selected gluten-free flours on the volatile profile of the corresponding crumbs through DHS-GC/MS. Selection of the most suitable flours and starches for the improvement of gluten-free bread crumb aroma.

6- Optimisation and validation of SHS-GC/QTOF, SPME-GC/QTOF and SAFE-GC/QTOF methodologies for the quantification of volatile compounds in gluten-free flours and starches.

7- Optimisation and validation of SPME-GC/QTOF methodology for the quantification of volatile compounds in gluten-free bread crusts. Selection of the most suitable flours and starches for the improvement of gluten-free bread crust aroma.

8- Proposal of different mixtures of flours and starches for the improvement of the aroma of the whole bread. Selection of the final recipe.

III. Structure

Structure

The research carried out during this Doctoral Thesis has given rise to several scientific publications that fulfil the objectives proposed and whose content is presented in the following sections.

Introduction prompted two reviews, which were the result of an extensive search of information learnt during this Doctoral Thesis. However, the introduction contains just certain parts of these reviews which were adapted to the introduction structure.

- Pico, J., Bernal, J., & Gómez, M. (2015). Wheat bread aroma compounds in crumb and crust: A review. *Food Research International*, 75, 200-215.
- Pico, J., Gómez, M., Bernal, J., & Bernal, J.L. (2016). Analytical methods for volatile compounds in wheat bread: A review. *Journal of Chromatography A*, 1428, 55-71.

Section 1: Solvent extraction methodologies for the analysis of volatile compounds in bread crumb

It consists on the development and validation of a solvent extraction methodology for the quantification of volatile compounds in breads with low fat content through the hydrolysis of the lipids with lipases. It was developed as an alternative to the classical SAFE methodology, which presented problems of losses of volatile compounds during the handle of the glass device (low recovery percentages) (Engel et al., 1999) as well as unsuitable intermediate precision % RSD (Havemose et al., 2007; Majcher & Jeleń, 2009). Moreover, the glass device is fragile and tricky to clean. Once the "lipases methodology" was developed, optimised and validated, its analytical characteristics were compared with those of a developed SAFE methodology. This section includes the following articles and meets the objective 1:

- Pico, J., del Nozal, M. J., Gómez, M., & Bernal, J.L. (2016). An alternative method based on enzymatic fat hydrolysis to quantify volatile compounds in wheat bread crumb. *Food Chemistry*, *206*, 110-118.
- Pico, J., Oduber, F., Gómez, M., & Bernal, J. Analytical feasibility of a SAFE method for aroma analyses in bread crumb. *Under Review*.

Section 2: Approaches for achieving accurate analyses of volatile compounds in bread dough and crumb

It includes preliminary studies that were necessary in order to obtain accurate results. Bread samples were not usually analysed at the same moment of being baking; thus, freezing before aroma analyses has been a common practice in the literature (Bianchi, Careri, Chiavaro, Musci, & Vittadini, 2008; Luning, Roozen, Moëst, & Posthumus, 1991; Paraskevopoulou, Chrysanthou, & Koutidou, 2012). However, none of these articles have checked if there were losses or changes of volatile compounds during freezing. The study of the "frozen storage time" with the "lipases method" and SHS-GC/MS was made in order to select the maximum freezing time that the volatile profile remained unchangeable. On the other hand, since the doughs evolved after fermentation due to the yeast action, it was necessary to inhibit the fermentation evolution in order to analyse the dough's volatile profile at the desired fermentation time. Mercuric chloride has been the most common option for fermentation inhibition (Martínez-Anaya, Torner & Benedito de Barber, 1990), but it is toxic and harmful for humans and its use will be forbidden in a near future (Pu et al., 2014). Thus, a non-toxic alternative for the inhibition of fermentation evolution was researched by SPME-GC/QTOF. This section includes the following articles, and it also complies objectives 2 and 3:

- Pico, J., Martínez, M. M., Bernal, J., & Gómez, M. (2017). Impact of frozen storage time on the volatile profile of wheat bread crumb. *Food Chemistry*, *232*, 185-190.
- Pico, J., Bernal, J., del Nozal, M. J., & Gómez, M. (2017). Inhibition of fermentation evolution in bread doughs for aroma analyses. *Flavour and Fragance Journal*, *32*, 461-469.

Section 3: Generation of volatile compounds in gluten-free bread doughs and crumbs. Selection of the most suitable flour/ starch for the improvement of the gluten-free bread crumb aroma

It presents firstly the study with the lipases method of the evolution of volatile compounds of gluten-free bread (made with corn starch) from dough to crumb at different fermentation times. Since it was proven, for the first time in the literature of gluten-free breads, that there were changes in the concentration of the volatile compounds during fermentation as well as from the fermentation to baking, the effect of different flours and starches in the volatile profile of the corresponding doughs and crumbs was screening using SHS-GC/MS. Once certain flours and starches were selected, the volatile profiles of the corresponding crumbs were evaluated by DHS-GC/MS in order to select the most suitable flours and starches for the improvement of the gluten-free bread crumb aroma. This section is based on the following articles and meets with objectives 4 and 5:

- Pico, J., Martínez, M. M., Bernal, J., & Gómez, M. (2017). Evolution of volatile compounds in gluten-free bread: From dough to crumb. *Food Chemistry*, 227, 179-186.
- Pico, J., Bernal, J. L., & Gómez, M. (2017). Influence of different flours and starches on gluten-free bread aroma. *Journal of Food Science and Technology*, 54, 1433-1441.

- Pico, J., Hansen, Å.S., Petersen, M. A. (2017). Comparison of the volatile profiles of the crumb of gluten-free breads by DHE-GC/MS. *Journal of Cereal Science*, 76, 280-288.

Section 4: Volatile compounds in gluten-free flours and starches and the importance of the aroma of gluten-free bread crusts. Selection of the most suitable flour/ starch for the improvement of the gluten-free bread crust aroma

Since in Section 3 there were differences in the volatile profiles of the different gluten-free bread crumbs, in Section 4 the volatile compounds from different gluten-free flours and starches were examined by a developed and validated SPME-GC/QTOF method in order to highlight if these volatile compounds were originated during fermentation and baking or came from the flour or starch itself, since there were no report in the literature of gluten-free breads. Moreover, as the volatile compounds of the different gluten-free bread crumbs were the same but in different concentrations, it was suggested that great differences between gluten-free breads should be found in the crust. Thus, a SPME-GC/QTOF method for the semi-quantitative and quantitative analysis of volatile compounds in bread crust was developed and validated. It was applied to the quantification of different gluten-free bread crust volatile compounds in order to select the most suitable flours and starches for the improvement of the gluten-free bread crust aroma. This section is based on the following articles and meets with the objectives 6 and 7:

- Pico, J., Tapia, J., Bernal, J., & Gómez, M. (2017). Comparison of different extraction methodologies for the analysis of volatile compounds in gluten-free flours and corn starch by GC/QTOF. *Food Chemistry,In press.* http://dx.doi.org/10.1016/j.foodchem.2017.06.157

- Pico, J., Antolín, B., Román, L., Gómez, M., & Bernal, J. Analysis of volatile compounds in gluten-free bread crusts with an optimised and validated SPME-GC/QTOF methodology. *Food Research International*, 106, 686-695.

Section 5: Study of the most appropriate mixture of flours and starches for the improvement of the final aroma of gluten-free bread.

Taking into consideration the conclusions extracted from the crumb and crust studies in Section 3 and 4, in Section 5 different mixtures of the selected flours and starches were proposed in order to obtain gluten-free breads with an improved aroma. The final recipe was selected through the volatile profiles analysed by SPME-GC/QTOF. This section gives rise to the following article, and it also complies objective 8:

- Pico, J., Antolín, B., Román, L., Bernal, J., & Gómez, M. Selection of the most suitable mixture of flours and starches for the improvement of gluten-free breads through their volatile profiles. *Under Review*.

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V. Section 1:

Solvent extraction methodologies for the analysis of volatile compounds in bread crumb

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Analytical Methods

An alternative method based on enzymatic fat hydrolysis to quantify volatile compounds in wheat bread crumb

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ABSTRACT

An alternative method to quantify 40 volatile compounds in wheat bread crumb is proposed. It consists of a Soxhlet extraction with a mixture of dichloromethane and diethyl ether containing lipases and a subsequent concentration with Vigreux column. It is the first time that lipases are added to transform the fat into free fatty acids and glycerol, which elute at the end of the chromatogram after the analytes, avoiding problems in the chromatography due to fat residues, such as dirtiness in the injector, column clogging or overlapping peaks. The extract is most easily analysed by GC/MS, using a standard addition method to correct matrix effect. The method was fully validated, with extraction efficiencies between 70% and 100% and precision RSD lower than 15%. The method was applied to a commercial crumb, with acetoin, phenylethyl alcohol and acetic acid as highly abundant compounds, which are considered main volatiles in crumb.

1. Introduction

Bread is one of the most widely consumed foods in the world. It is made with a mixture of flour, water, yeast and salt, all in the right proportions, which is then kneaded, fermented and baked. There are different properties that define its quality, such as volume, texture, colour and flavour, although the aroma of bread is considered essential to its approval by consumers. Analyses of the volatile fraction of bread are very important in order to obtain breads with pleasant smells and also in order to understand the baking processes better.

It is necessary, therefore, to develop analytical methods that allow fast, less expensive and accurate analyses of volatile compounds. There have so far usually been two options to analyse the volatile fraction of breads (Pico, Gómez, Bernal, & Bernal, 2016): solvent extraction methods (Kirchhoff & Schieberle, 2001; Moskowitz, Bin, Elias, & Peterson, 2012; Onishi, Inoue, Araki, Iwabuchi, & Sagara, 2011; Peterson & Jiang, 2013; Rychlik & Grosch, 1996; Zehentbauer & Grosch, 1998) and head space (HS) analyses (Birch, Petersen, Arneborg, & Hansen, 2013; Birch, Petersen, & Hansen, 2013; Maeda et al., 2009; Makoul et al., 2015; Poinot et al., 2007; Ruiz, Quílez, Mestres, & Guasch, 2003). Both methods involve the gas chromatography technique, very often equipped with a mass spectrometry detector (GC/MS). The head space method implies a fast sample treatment with no handling of the matrix, but it contains some disadvantages: HS methods include only the analysis of very volatile compounds (Birch, Petersen, & Hansen, 2014), and in order to obtain high reproducibility, HS techniques require the precise control of many parameters (Birch et al., 2013). Moreover, the amount of isolated volatile fraction is at times so small that important volatile compounds present in bread in low concentrations give no detector signal. Solvent extraction methodologies overcome these drawbacks, but they are time-consuming; the greater the number of steps in sample treatment, the greater the likelihood of analyte losses. Classical solvent extraction techniques have involved extracting the volatile fraction, normally with Soxhlet, and the subsequent concentration with Vigreux columns. However, Soxhlet extraction employs organic solvents that also extract fat from the

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Abbreviations: DAGs, diacylglycerides; GC/MS, gas chromatography/mass spectrometry; HS, head space; HVT, high vacuum transfer; LOD, limit of detection; LOQ, limit of quantification; MAGs, monoacyglycerides; MSA, method of standard additions; Qi, qualifier ion i; R^2, determination coefficient; RSD%, relative standard deviation; LipCL, Lipozyme CALB L®; LipPT, Lipase Palatase 20000 L®; LipTL, Lipozyme TL 100 L®; SAFE, Solvent Assisted Flavour Evaporation; SDE, Simultaneous Steam Distillation Extraction; T, target ion; TAGs, triacylglycerides; VS, Vacuum Sublimation.

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bread matrix. Fat could dirty the injector and the GC column and also interact with volatile substances, affecting the recovery percentages (Engel, Bahr, & Schieberle, 1999). Different methods to isolate volatile from non-volatile compounds like fat have emerged: Simultaneous Steam Distillation Extraction (SDE) (Lin, Hsieh, Liu, Lee, & Mau, 2009), Vacuum Sublimation (VS) (Schieberle & Grosch, 1987) and the last method, Solvent Assisted Flavour Evaporation (SAFE) (Engel et al., 1999). Within this context, the use of lipases could represent an alternative to dealing with bread fat. Lipases are enzymes that hydrolyse triacylglycerides (TAGs), diacylglycerides (DAGs) and monoacylglycerides (MAGs) into free fatty acids and glycerol (Murty, Bhat, & Muniswaran, 2002), which are more volatile than TAGs, DAGs and MAGs of fat.

The purpose of the study was to develop and validate an alternative method to analyse volatile compounds in wheat bread crumb by means of lipases, in order to transform the fat by hydrolysis into free fatty acids and glycerol that could elute after the analytes, avoiding chromatographic interferences and instrument dirtiness. As far as we know, this is the first time that lipases have been employed in food aroma analyses with the aim of transforming the fat to avoid the subsequent difficulties in chromatographic determinations.

2. Materials and methods

2.1. Materials, reagents and standards

Dichloromethane and diethyl ether, employed in Soxhlet extraction, were purchased from LAB-SCAN (Gliwice, Poland) and Panreac (Barcelona, Spain), respectively. Methanol used in the standard preparation was obtained from VWR Chemicals (Fontenay-sous-bois, France). Liquid nitrogen used to ground the wheat bread crumb was purchased from Carburos Metálicos (Barcelona, Spain). Enzymes Palatase 20000 L®, Lipozyme TL 100 L® (both from *Aspergillus oryzae*; hydrolyse 1 and 2 bonds) and Lipozyme CALB L® (from *Aspergillus niger*; hydrolyses 1, 2 and 3 bonds) were the lipases tested to hydrolyse fat in the Soxhlet extraction and were all kindly provided from Novozymes (Bagsvaerd, Denmark). The standards employed in the experimental study were all neat and were supplied by Sigma–Aldrich (Steinheim, Germany). They are listed in Table 1 (see Section 2.4).

Table 1
Volatile compounds studied in the validated method in order of elution. Target (T) and qualifier (Q_1, Q_2, +Q) ions employed for each compound are given in the table. The numbering used for the peaks in Fig. 2 is also given in the last column.

Volatiles	T	Q_1	Q_2	+Q	Peak label
2,3-Butanedione	43	86	15	42	1
1-Propanol	31	42	59	60	2
2-Methyl-1-propanol	43	41	74	55	3
Hexanal	44	56	72	82	4
3-Penten-2-ol	71	43	53	86	5
2-Methyl-1-butanol	57	41	70	29	6
3-Methyl-1-butanol	55	70	41	57	7
R-(+)-limonene	68	93	79	107	8
Ethyl hexanoate	88	99	43	60	9
1-Pentanol	42	55	70	91	10
Acetoin	45	88	27	15	11
2-Octanone	58	71	85	128	12
1-Hydroxy-2-propanone	43	31	74	29	13
Ethyl lactate	45	75	29	19	14
1-Hexanol	56	43	69	84	15
Nonanal	57	41	70	98	16
Acetic acid	45	60	15	29	20
1-Octen-3-ol	57	72	43	85	18
Methional	48	104	76	61	19
Furfural	96	39	29	67	21
Ethyl octanoate	88	101	127	57	17
2-Ethyl-1-hexanol	57	41	70	83	22
Benzaldehyde	106	105	77	51	23
2,3-Butanediol	45	57	29	75	24
2-(E)-nonenal	70	55	41	83	25
Isobutyric acid	43	41	73	27	26
5-Methyl-2-furaldehyde	110	109	53	81	27
1,2-Propanediol	45	43	61	29	28
Butyric acid	60	73	42	27	29
Butyrolactone	42	28	86	56	30
Phenylacetaldehyde	91	120	92	65	31
Furfuryl alcohol	98	81	53	69	32
2-Methylbutanoic acid	57	74	87	41	33
3-Methylbutanoic acid	60	43	87	39	34
1,3-Butanediol	43	45	57	72	35
2,4-(E,E)-decadienal	81	67	95	152	36
Hexanoic acid	60	73	87	41	37
Benzyl alcohol	79	108	91	51	38
Phenylethyl alcohol	91	122	65	77	39
4-Vinylguaiacol	150	135	107	77	40

2.2. Sample description

The method was developed and validated with the crumb of a commercial loaf of wheat bread on the Spanish market. The label showed the ingredients to be wheat flour, water, salt, yeast and flour improver (wheat flour, anti-caking agent (E-170), emulsifier (E-472e), antioxidant (E-300) and enzymes). The loaf of bread weighed 450 g, of which around 130 g were crumb. Sampling is detailed in Section 2.5.

2.3. Preparation of standard solutions

Stock solutions (10,000 mg L^{-1}) of each volatile compound (listed in Table 1) were prepared in methanol. The working solutions were prepared from the mix of stock solutions as required and methanol was used to dilute. All the solutions were stored in a freezer at −21 °C.

2.4. GC–MS instrumentation and chromatographic conditions

GC–MS analyses were performed on a 7890A gas chromatograph (GC) coupled to a 5975C mass spectrometer (MS) detector, which was equipped with a 7683B automatic injector and MS ChemStation 5975C software, all from Hewlett Packard (Palo Alto, California, USA). Separation was achieved on a polar ZB-Wax column (100% polyethylene glycol, 60 m × 0.25 mm ID × 0.25 μm) obtained from Phenomenex (Torrance, California, USA). The GC was operated under programmed temperature conditions ranging from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, after which the temperature was increased to 114 °C (3 min) at 6 °C/min, and then to 136 °C (0 min) at 1.5 °C/min. Finally, the temperature was raised to 245 °C at 85 °C/min. This was held for 8 min in the case of standard solution injection, but for 25 min for wheat bread crumb samples (as explained in Section 3.1.1). Total run time was 48 min and 65 min, respectively. An injection volume of 1 μL was employed with the autosampler in pulsed splitless mode. The inlet temperature was set at 250 °C and the carrier gas was Helium supplied by Carburos Metálicos (Barcelona, Spain) at a flow rate of 1.1 mL/min. The interface, ion source and quadrupole temperatures were 250 °C, 230 °C and 150 °C, respectively. The MS scan parameters included a mass range of 15–350 m/z, operating in positive electron impact mode with ionisation energy of 70 eV. Analyses were performed with selected ion monitoring mode (SIM), with one target (T) and two qualifier ions (Q_1 and Q_2) for each volatile compound (see Table 1). The 40 analytes were identified and confirmed by a comparison of their retention times and mass spectra with standards and the Mass Spectra Library (Wiley 7N edition).

2.5. Sample preparation

2.5.1. Previous method (Method A)

First of all, the crumb was carefully separated from the crust. The loaf of bread was cut into slices of 5 cm and the ends were discarded since they contained little crumb. In each slice, the separation from crumb to crust was 1 cm in order to avoid contamination due to volatile compounds in the latter. Then the crumb was frozen with liquid nitrogen and ground in an Ika grinder with 5 intervals of 5 s each. 50 g of the frozen powder were inserted into the Soxhlet thimble and transferred to a Soxhlet apparatus. The extraction was accomplished with 300 mL of a mixture of diethyl ether/dichloromethane (2:1). Extraction was carried out for 5 h at 40 °C and the distillation flask was cooled to room temperature. The extract was concentrated to a final volume of 1 mL with a Vigreux column of 50 cm. The sample was immediately injected onto GC/MS.

2.5.2. Alternative method (Method B)

The sample treatment is modified in one step: 25 μL of lipase enzyme LipCL (Lipozyme CALB L®) are added to the mixture of 300 mL of diethyl ether/dichloromethane, prior to Soxhlet extraction. While solvent extraction is taking place, lipases are hydrolysing the fat at the same time.

2.6. Method validation

2.6.1. Accuracy: recovery percentages, matrix effect and extraction efficiency

When a crumb sample is spiked and processed, the chromatographic peak signals are affected not only by the efficiency of Soxhlet extraction but also by the possible matrix effect in the MS detector during ionisation. Therefore, the recovery percentage incorporates both matrix effect and extraction efficiency.

2.6.1.1. Recovery percentages (Methods A and B). Since there are no crumb samples without volatile compounds (blank), we spiked 50 g of frozen crumb powder with 10 mL of a standard concentration mix that could perceptibly increase these peaks (3 mg L^{-1}). Next, both the spiked sample and the sample without spiking (to subtract the signal) were treated simultaneously by both Methods A and B, in order to evaluate the benefits of adding lipases to the extractant. The spiked sample, non-spiked sample and standard mix (concentrated at the same level as the standard in the spiked sample following treatment, that is, 10-fold) were prepared in duplicate and injected consecutively in triplicate ($n = 6$).

2.6.1.2. Matrix effect (Method B). In order to examine only the effect on the MS detector, a crumb sample was spiked after sample treatment. The extract obtained from the Soxhlet extraction was concentrated under 1 mL in a Vigreux column, spiked with 100 μL of a standard mix (300 mg L^{-1}) and finally made up to the volume. It was prepared in duplicate and injected in triplicate ($n = 6$).

2.6.1.3. Extraction efficiency (Method B). This parameter was evaluated by comparing the recovery percentages (which take into account the extraction efficiencies and the matrix effect, since the crumb is spiked before sample treatment) with the matrix effect values (which take into consideration only the effect of the MS detector, the extract being spiked after sample treatment). It evaluates the authentic efficacy of the Soxhlet extraction (Method B), without any increase or decrease in the chromatographic peak signal due to the matrix.

2.6.2. Limits of detection (LOD) and quantification (LOQ) (Method B)

These figures of merit were evaluated with the standards in the matrix (crumb sample). They were calculated by taking into consideration the area of analyte peaks from a spiked sample and the area of the noise from a solvent blank at the same retention time as that of the analyte peaks. Injection was made in triplicate ($n = 3$). The solvent employed to measure the noise was a mixture of diethyl ether/dichloromethane (2:1). As the crumb samples already contained large amounts of several of the analytes of interest, it was necessary to spike the sample with a standard that greatly increases the area (3 mg L^{-1}).

2.6.3. Linearity (Method B)

The linearity of the method was evaluated by calculating the regression line of the standards in the matrix (spiked crumb samples) and expressed by the determination coefficient (R^2), covering the concentration range of 10–1000 μg K^{-1}. A "*t* test" was carried out for each analyte to check the linearity of the method and the intercept interval was also verified so as to contain zero. The linear interval was studied with seven calibration points injected in triplicate ($n = 3$).

2.6.4. Precision: intra-day repeatability, inter-day repeatability and intermediate precision (Method B)

For intra-day repeatability, an extract of a spiked sample (final concentration of 600 μg K^{-1}) was injected in triplicate and the RSD (%) of each compound was calculated ($n = 3$). In terms of inter-day repeatability, the same extract of a spiked sample was injected in triplicate on three alternate days and RSD (%) was calculated ($n = 9$). Finally, to study intermediate precision, a spiked sample was prepared in duplicate by different analysts, as in Section 2.5, with the same conditions, and each sample was subsequently injected in triplicate, with a calculation finally being made of the RSD (%) ($n = 6$).

2.7. Volatile compound quantification: method of standard addition (MSA) (Method B)

When quantifying, it is necessary to take the sample matrix into consideration. This means using MSA, which is a precise method of quantification. We spiked seven crumb aliquots with standard mix concentrations ranging from 0.05 mg L^{-1} to 5 mg L^{-1}. Each point was injected in triplicate ($n = 3$).

3. Results and discussion

3.1. Development of sample preparation

3.1.1. Lipases: an alternative way to deal with bread fat

As can be seen in Fig. 1, when a crumb aliquot underwent sample treatment without added lipases (Method A, see Section 2.5.1), an accumulation of overlaid peaks appeared in the final part of the chromatogram (40.5–41.50 min, Fig. 1), causing interferences mainly with phenylethyl alcohol (peak b, Fig. 1). The accumulation of retained ions (peak a, Fig. 1), like m/z 73, was identified for the Mass Spectra Library as fatty acids (probably released from fragmentations in the MS detector). However, when any of the three enzymes were added (Method B, see Section 2.5.2), the accumulation of overlaid peaks disappeared but glycerol and fatty acids appeared. This indicated that the fat had been hydrolysed into free fatty acids and glycerol, which were more volatile than fat (peaks d and e, Fig. 1). By increasing the hold time of the final temperature of the GC temperature program (25 min), these free fatty acids and glycerol eluted in the final part of the chromatogram. In this way, they could not cause interference with volatile compounds that

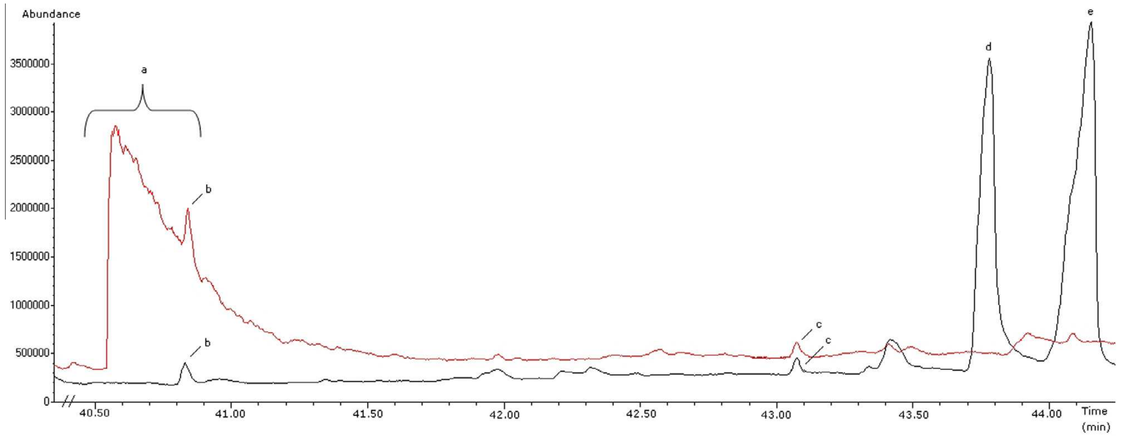

Fig. 1. Superposition of chromatograms of a sample without the addition of lipases (chromatogram in red) and with the addition of lipase Lipozyme CALB L (chromatogram in black). Only the zone with the interference of fat (in red) and the presence of glycerol and fatty acids (in black) are expanded. The volatile fraction is shown in Fig. 2. Peaks labels: a (fat interferences); b (phenylethyl alcohol); c (4-vynilguaiacol); d (glycerol); e (fatty acid). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eluted previously. Apart from the chromatographic interferences, it was observed that the oily extract from Method A caused visible dirtiness in the liner and septum from injector, with the possibility of column clogging. However, when lipases were added the extract was much less oily and the dirtiness in the injector was considerably reduced. We tested three different lipases specific to hydrolysis of ester bonds in glycerides. LipPT (Palatase 20000 L®) and LipTL (Lipozyme TL 100 L®) hydrolyse 1 and 3 bonds, while LipCL (Lipozyme CALB L®) hydrolyses 1, 2 and 3 bonds. Their activity at 40 °C (extraction temperature) is higher than 80%. The required amount was calculated on the basis of their lipase units (LU) per gram of glyceride. 50 μL per 100 g of bread crumb was deemed to be sufficient to hydrolyse most glycerides.

Moreover, when a solvent blank was injected immediately after the elution of a crumb extract obtained with Method A, there were peaks corresponding to fatty acids, indicating that they had been withheld in the GC column. In case of a crumb extract from Method B, when extending the hold time of the final temperature of the GC program 17 min more, the blank injected right after was completely clean (the retained fatty acids eluted in the added min of the chromatogram). Moreover, with the transformation of the fat into free fatty acids and glycerol, extraction efficiencies were demonstrated to be between 70% and 100% in all volatile compounds (see Section 3.2.1).

With the use of LipTL or LipPT, the peak corresponding to glycerol (absent in samples without enzymatic treatment and indicative of hydrolysis) was very small; consequently, the rates of hydrolysis would have been low. Moreover, with LipTL there was an increase also in 1,2-propanediol (a slight peak in a sample without enzymatic treatment). However, when using LipCL the glycerol peak increased a great deal (more than 6 times) since it hydrolyses three bonds and acts more efficiently not only in 1,3-DAGs but also in TAGs. Moreover, it is proven that it can work in inert organic solvents. There was no formation of new volatile compound peaks, no increased 1,2-propanediol, whilst the existing signals of the volatile compounds being studied neither diminished nor increased considerably. Therefore, LipCL was the enzyme chosen.

3.1.2. Selection of Soxhlet extractant

The extractant most widely employed in Soxhlet extraction for volatile compounds in bread has been dichloromethane (Kirchhoff & Schieberle, 2001; Onishi et al., 2011; Rychlik & Grosch, 1996; Zehentbauer & Grosch, 1998), although reports exist of the use of diethyl ether to extract acidic compounds and dichloromethane for basic and neutral compounds (Zehentbauer & Grosch, 1998). Two 50 g aliquots of a spiked sample underwent sample treatment of 5 h extraction at 40 °C (Method B), one with 300 mL of dichloromethane and the other with 300 mL diethyl ether. Recovery percentages of 2,3-butanedione, 1-propanol, 1-pentanol, 2-methyl-1-butanol, 3-methyl-1-butanol, hexanal, 2-octanone and ethyl hexanoate, were much lower in dichloromethane, not reaching 50% (3.2% for 1-propanol and 8.6% for hexanal). Those of limonene, 1-hexanol, benzaldehyde, 2,3-butanediol, butyrolactone and phenylacetaldehyde were slightly lower in diethyl ether. The remainder were either similar in both solvents or approximately 10% higher in diethyl ether, with the exception of 2-methylbutanoic acid and 3-methylbutanoic acid, which in diethyl ether gave recoveries of close to 100% while in dichloromethane they were around 65%. Therefore, a mixture of diethyl ether/dichloromethane is deemed appropriate. The amount of diethyl ether should be a great deal higher, principally to increase the recovery percentages of 2,3-butanedione, 1-propanol, 1-pentanol, 2-methyl-1-butanol, 3-methyl-1-butanol, hexanal, 2-octanone and ethyl hexanoate. The boiling point of diethyl ether is lower than that of dichloromethane; therefore, the larger proportion of diethyl ether lowers the boiling point of the mixture, with a reduction in the loss of the most volatile compounds. A mixture of diethyl ether/dichloromethane in the proportion 2:1 provided the best extraction efficiencies, as can be seen in Table 2.

3.1.3. Selection of the Soxhlet extraction time

In order to improve the recoveries that did not reach 100%, longer extraction times were tested (Method B). With a 15 h extraction, all recoveries diminished considerably compared with 5 h extraction, probably due to losses resulting from volatility. Finally, a reduction in extraction time to 2.5 h was also attempted. However, recoveries also decreased compared with 5 h extraction time, which was due, in all likelihood, to extraction not having finished. Therefore, 5 h was chosen as Soxhlet extraction time.

3.2. Method validation

3.2.1. Accuracy: recovery percentages, matrix effect and extraction efficiency

The advantages of the use of lipases were proved by the higher recovery percentages of Method B (with lipases) versus Method A

Table 2
Recovery percentages, matrix effect and extraction efficiencies of each volatile compound studied in the validated method. Extraction efficiencies were obtained by correcting the recovery percentages with the matrix effect values.

Volatiles	Recovery percentage (%)	Matrix effect (%)	Extraction efficiency (%)
2,3-Butanedione	57.9	43.7	114.2
1-Propanol	65.1	96.2	69.0
2-Methyl-1-propanol	99.9	129.0	70.9
Hexanal	70.1	53.2	116.9
3-Penten-2-ol	121.9	117.2	104.7
2-Methyl-1-butanol	80.9	112.9	67.9
3-Methyl-1-butanol	111.0	119.8	91.2
R-(+)-limonene	58.0	94.7	63.4
Ethyl hexanoate	70.8	98.6	72.1
1-Pentanol	48.2	37.3	110.8
Acetoin	142.7	142.3	100.5
2-Octanone	65.5	92.9	72.6
1-Hydroxy-2-propanone	134.1	143.9	90.3
Ethyl lactate	63.5	98.6	65.0
1-Hexanol	44.7	43.5	101.2
Nonanal	69.5	103.3	66.2
Acetic acid	98.5	87.9	110.6
1-Octen-3-ol	75.8	89.5	86.3
Methional	56.5	93.4	63.2
Furfural	93.8	105.8	88.1
Ethyl octanoate	122.9	111.5	111.4
2-Ethyl-1-hexanol	68.0	79.7	88.3
Benzaldehyde	55.0	99.4	55.5
2,3-Butanediol	96.5	99.3	97.2
2-(E)-nonenal	104.0	107.1	96.9
Isobutyric acid	87.8	78.9	108.9
5-Methyl-2-furaldehyde	76.5	103.8	72.7
1,2-Propanediol	134.6	132.0	102.5
Butyric acid	109.9	104.9	105.0
Butyrolactone	88.0	73.3	114.7
Phenylacetaldehyde	79.0	85.8	93.2
Furfuryl alcohol	116.8	101.1	115.7
2-Methylbutanoic acid	107.6	126.5	81.1
3-Methylbutanoic acid	96.0	120.8	75.2
1,3-Butanediol	99.2	105.0	94.2
2,4-(E,E)-decadienal	70.3	107.4	62.9
Hexanoic acid	103.9	134.6	69.3
Benzyl alcohol	89.4	112.8	76.6
Phenylethyl alcohol	96.3	100.1	96.2
4-Vinylguaiacol	98.1	121.5	76.6

(the previous method), as it is indicated in Table S1 (see supplementary data). The improvement in the recovery percentages with the use of Method B was demonstrated even more in the case of acids, probably due to their interaction with the fat that hinders their extraction in case of Method A. Thus, it is shown that the fat not only causes interferences to the volatile compounds but also affects their recovery percentages. The rest of the validation was carried on with the proposed alternative sample treatment, Method B.

Since the recovery percentages of Method B were in the interval of 37.4–143.9% (Table 2), a study was made of possible matrix effect diminishing or increasing the signal during ionisation. The recovery percentages were affected by the sample treatment (principally, Soxhlet extraction) but also by ionisation in the MS detector. As can be seen in Table 2, the matrix effect was verified, since there were 10 volatile compounds displaying a considerable suppression of ionisation (1-octen-3-ol, acetic acid, phenylacetaldehyde, 2-ethyl-1-hexanol, isobutyric acid, butyrolactone, hexanal, 2,3-butanedione, 1-hexanol and 1-pentanol), and 13 volatile exhibiting a gain in ionisation (1-hydroxy-2-propanone, acetoin, hexanoic acid, 1,2-propanediol, 2-methyl-1-propanol, 2-methylbutanoic acid, 4-vinylguaiacol, 3-methylbutanoic acid, 3-methyl-1-butanol, 2-methyl-1-butanol, 3-penten-2-ol, benzyl alcohol and ethyl octanoate). 17 of the 40 compounds did not show a valuable matrix effect. Therefore, extraction efficiency values, rather than recovery percentages, give us a real idea of the quality of Soxhlet extraction in terms of the solvent and extraction time. By adjusting the recovery percentages with the matrix effects, extraction efficiencies were between 55.5% and 116.9%. As is shown in Table 2, 22 of 40 volatile compounds displayed extraction efficiencies close to 100%, while 17 compounds were around 70%, with benzaldehyde exhibiting an extraction efficiency of 55.5%. Therefore, Soxhlet extraction was optimised and the matrix effect proven, whilst quantification on the matrix was required (Sections 2.7 and 3.3).

3.2.2. Limits of detection (LOD) and quantification (LOQ)

As is shown in Table 3, LODs in the matrix were within the range of 0.31–33.2 μg K^{-1} and LOQs in the range of 1.03–110.8 μg K^{-1}.

3.2.3. Linearity

Table 3 shows that all the calibration curves displayed good linear regression in the matrix. R^2 were between 0.9901 and 0.9988 in the range of 10–1000 μg K^{-1}. The intercept interval contained zero in all cases and the “*t* test” was successful; therefore, the linearity of the method is proven.

3.2.4. Precision: intra-day repeatability, inter-day repeatability and intermediate precision

Intra-day repeatability, inter-day repeatability and intermediate precision were evaluated in the matrix (see Table 3). Intra-day repeatability RSD% ranged from 0.02% to 9.67%, whereas inter-day repeatability RSD% was between 3.0 and 13.7%. Finally, intermediate precision RSD% went from 0.14% to 8.9%. Regarding these three terms of precision, RSD% did not exceed 15%.

3.3. Sample quantification: method of standard addition (MSA)

The results for the volatile compounds under study are shown in Table 4 (given in μg K^{-1}) and the corresponding chromatogram in Fig. 2. There was no creation or reduction of volatile compounds between the sample analysed without the use of enzymes (Method A) and the sample that contained lipases to hydrolyse the fat that was present (Method B). As can be seen in Table 4, the wheat bread crumb sample is characterised by alcohols, ketones, aldehydes, acids and small quantities of esters, which is in agreement with those reported by Birch, Petersen, Arneborg, et al. (2013) in wheat bread crumb. DalíAsta et al. (2013) reported that the most characteristic compounds in crumb were alcohols from yeast fermentation. In the crumb sample analysed, 14 of the existent 38 volatile compounds were alcohols that come from fermentation (36.8%).

Moreover, there were small quantities of terpenes like limonene, which is considered a minor compound in crumb with a citrus odour (Ruiz et al., 2003), normally added as a flavour additive (Seitz, Chung, & Rengarajan, 1998). Ethyl hexanoate and ethyl lactate were absent, while ethyl octanoate was the least abundant volatile compound in the sample (1.93 μg K^{-1}). These esters come from reactions in the yeast cell catalysed by acetyltransferases, and could be important in crumb aroma due to their fruity odour (Birch et al., 2014). However, the low concentration or absence of these esters is not surprising since small esters are lost during baking (Birch, Petersen, Arneborg, et al., 2013, Birch et al., 2013).

The most abundant compounds were 3-hydroxy-2-butanone (acetoin, 409.6 μg K^{-1}), 3-penten-2-ol (374.4 μg K^{-1}), 4-vinylguaiacol (324.7 μg K^{-1}), phenylethyl alcohol (302.0 μg K^{-1}),

Table 3
Limits of detection (LOD), limits of quantification (LOQ), linearity and precision (intra-day repeatability, inter-day repeatability and intermediate precision) parameters of each volatile compound studied in the validated method.

Volatiles	LOD (μg K^{-1})	LOQ (μg K^{-1})	R^2	Intra-day repeatability RSD%	Inter-day repeatability RSD%	Intermediate precision RSD%
2,3-Butanedione	8.05	26.8	0.9984	1.5	5.0	1.8
1-Propanol	0.76	2.55	0.9971	3.2	4.8	2.4
2-Methyl-1-propanol	5.03	16.8	0.9976	5.0	7.8	8.9
Hexanal	2.61	8.71	0.9988	0.02	5.0	6.7
3-Penten-2-ol	0.37	1.24	0.9975	5.8	5.2	5.6
2-Methyl-1-butanol	0.90	3.00	0.9947	6.2	7.4	1.6
3-Methyl-1-butanol	1.43	4.78	0.9976	0.08	5.2	6.6
R-(+)-limonene	0.83	2.77	0.9985	6.1	6.9	4.1
Ethyl hexanoate	0.83	2.77	0.9977	5.9	6.4	7.8
1-Pentanol	7.81	26.0	0.9921	9.7	12.9	5.8
Acetoin	0.47	1.56	0.9946	6.2	5.7	2.2
2-Octanone	0.57	1.90	0.9957	7.7	7.6	4.6
1-Hydroxy-2-propanone	4.46	14.9	0.9971	5.0	5.7	7.5
Ethyl lactate	0.48	1.60	0.9964	5.4	6.6	7.8
1-Hexanol	0.69	2.32	0.9918	6.2	6.9	7.3
Nonanal	7.16	23.9	0.9932	1.7	12.7	0.14
Acetic acid	4.57	15.3	0.9901	1.6	5.3	8.0
1-Octen-3-ol	1.96	6.52	0.9951	6.8	7.6	1.6
Methional	1.55	5.17	0.9959	4.6	7.2	7.6
Furfural	1.03	3.44	0.9956	5.0	6.5	0.56
Ethyl octanoate	0.31	1.03	0.9939	5.3	13.7	5.0
2-Ethyl-1-hexanol	2.87	9.56	0.9924	5.0	8.7	7.5
Benzaldehyde	0.89	2.97	0.9972	5.1	7.0	3.7
2,3-Butanediol	2.23	7.44	0.9903	4.7	8.5	3.7
2-(E)-nonenal	2.38	7.93	0.9960	4.4	8.3	5.4
Isobutyric acid	2.09	6.95	0.9912	4.1	10.9	1.0
5-Methyl-2-furaldehyde	3.39	11.3	0.9929	4.5	7.0	4.2
1,2-Propanediol	0.59	1.98	0.9943	7.4	10.8	0.66
Butyric acid	2.26	7.55	0.9916	4.5	9.5	2.3
Butyrolactone	1.20	4.01	0.9913	4.0	8.0	7.5
Phenylacetaldehyde	1.25	4.16	0.9924	0.95	10.6	7.3
Furfuryl alcohol	2.02	6.73	0.9903	4.7	8.3	8.5
2-Methylbutanoic acid	1.93	6.43	0.9919	4.0	8.4	2.6
3-Methylbutanoic acid	0.69	2.30	0.9901	4.5	8.2	0.88
1,3-Butanediol	9.86	32.9	0.9968	3.1	3.0	2.7
2,4-(E,E)-decadienal	33.2	110.8	0.9924	0.41	6.9	5.0
Hexanoic acid	3.24	10.8	0.9903	4.2	8.0	8.7
Benzyl alcohol	4.22	14.1	0.9944	7.0	8.5	2.1
Phenylethyl alcohol	3.41	11.4	0.9904	4.7	6.4	3.4
4-Vinylguaiacol	3.03	10.1	0.9966	5.0	5.5	3.2

acetic acid (275.8 μg K^{-1}), 2-methylpropanoic acid (isobutyric acid, 274.4 μg K^{-1}), hexanoic acid (244.8 μg K^{-1}) and 2,3-butanediol (244.7 μg K^{-1}). Acetoin and phenylethyl alcohol have already been reported as responsible for crumb aroma, and have been considered major volatile compounds quantified in wheat bread crumb (Birch et al., 2013; Ruiz et al., 2003). Acetoin comes from glycolysis of pyruvic acid during fermentation (Martínez-Anaya, 1996), but it can also be formed during Maillard reactions (Poinot et al., 2010). Phenylethyl alcohol is a fusel alcohol that is derived from the amino acid phenylalanine during the Ehrlich pathway by yeast fermentation (Gassenmeier & Schieberle, 1995; Paraskevopoulou, Chrysanthou, & Koutidou, 2012), but it can also be generated from phenylalanine as a Strecker degradation product during Maillard reactions (Moskowitz et al., 2012). However, in crumb both are expected to originate during fermentation. The great abundance of 2,3-butanediol is related with the huge amount of acetoin, since 2,3-butanediol is derived from the latter during the glycolysis process (Drapon & Richard-Molard, 1979). 2,3-butanedione also derives from acetoin via glycolysis (Drapon & Richard-Molard, 1979), but it has been considered a major volatile compound in crumb (Ruiz et al., 2003). Birch et al. (2013) reported that 2,3-butanedione could also originate from the oxidative decarboxylation of 2-acetolactate, and could furthermore be enzymatically reduced to acetoin or 2,3-butanediol by yeast. In view of the glycolysis of pyruvic acid (2,3-butanediol and 2,3-butanedione derive from acetoin) or the oxidative decarboxylation of 2-acetolactate (acetoin and 2,3-butanediol derive from 2,3-butanedione), acetoin is then the most abundant compound in the sample analysed, followed by 2,3-butanediol and finally 2,3-butanedione. The richness of phenylacetaldehyde (104.1 μg K^{-1}) should also be related with the large amount of phenylethyl alcohol, since both stem from the amino acid phenylalanine by the Ehrlich pathway in fermentation (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). Phenylacetaldehyde is the most abundant aldehyde in the sample, followed by 2,4-(E,E)-decadienal (51.9 μg K^{-1}), benzaldehyde (49.3 μg K^{-1}), furfural (44.2 μg K^{-1}), nonanal (37.0 μg K^{-1}), 5-methy-2-furaldehyde (17.2 μg K^{-1}) and, finally, 2-(E)-nonenal (9.0 μg K^{-1}). 2,4-(E,E)-decadienal, nonanal and 2-(E)-nonenal are lipid oxidation compounds that are not as abundant as aldehydes coming from the Ehrlich pathway, due to the fact that the addition of yeasts reduces the level of oxygen used by lipoxygenase enzymes to generate these aldehydes from the oxidation of lipids (Poinot et al., 2008). Heterocyclic aldehydes, such as furfural (from fermentation) or benzaldehyde (from fermentation or lipid oxidation), should be present also in crust because of Maillard reactions. In fact, 5-methyl-2-furaldehyde has been normally reported in the crust (Schieberle & Grosch, 1985, 1987), thus it is likely to have been transferred to the crumb in the sample analysed. Likewise, there are only two more heterocyclic compounds present in the analysed crumb sample (furfuryl alcohol or butyrolactone) that should also be present in crust, since they are minor volatiles quantified in crumb (Ruiz et al., 2003).

Table 4
Quantification of volatile compounds in the wheat bread crumb sample by the Method of Standards Addition (MSA) with "Method B". Results are given in $\mu g\ K^{-1}$. The standard deviations (SD) in $\mu g\ K^{-1}$ are also given.

Volatiles	$\mu g\ K^{-1}$	SD
2,3-Butanedione	111.9	2.0
1-Propanol	186.1	4.5
2-Methyl-1-propanol	187.3	16.6
Hexanal	52.1	3.5
3-Penten-2-ol	374.3	20.8
2-Methyl-1-butanol	114.7	1.9
3-Methyl-1-butanol	149.6	9.9
R-(+)-limonene	54.4	2.2
Ethyl hexanoate	nd[a]	nd[a]
1-Pentanol	27.8	1.6
Acetoin	409.6	9.2
2-Octanone	8.94	0.41
1-Hydroxy-2-propanone	98.8	7.4
Ethyl lactate	nd[a]	nd[a]
1-Hexanol	81.1	5.9
Nonanal	37.0	0.1
Acetic acid	275.8	22.1
1-Octen-3-ol	34.3	0.6
Methional	50.2	3.8
Furfural	44.2	0.3
Ethyl octanoate	1.93	0.10
2-Ethyl-1-hexanol	80.0	6.0
Benzaldehyde	49.3	1.8
2,3-Butanediol	244.7	9.0
2-(E)-nonenal	9.04	0.49
Isobutyric acid	274.4	2.9
5-Methyl-2-furaldehyde	17.2	0.7
1,2-Propanediol	197.0	1.3
Butyric acid	109.8	2.6
Butyrolactone	84.1	6.3
Phenylacetaldehyde	104.1	7.7
Furfuryl alcohol	80.1	6.8
2-Methylbutanoic acid	128.3	3.3
3-Methylbutanoic acid	72.0	0.6
1,3-Butanediol	13.7[b]	0.4
2,4-(E,E)-decadienal	51.9[b]	2.6
Hexanoic acid	244.8	21.4
Benzyl alcohol	24.4	0.5
Phenylethyl alcohol	302.0	10.2
4-Vinylguaiacol	324.7	10.3

[a] Not detectable (not present or below the limits of detection).
[b] Values below the limits of quantification.

The wealth of acids (acetic, isobutyric and hexanoic) and less abundant 2-methylbutanoic acid ($128.3\ \mu g\ K^{-1}$), butyric acid ($109.8\ \mu g\ K^{-1}$) and 3-methylbutanoic acid ($72.0\ \mu g\ K^{-1}$), most likely results from the addition of sourdough during commercial wheat bread elaboration. The interaction of LABs and yeasts during fermentation constitutes an increase in the production of organic acids from C3 to C6, as Martínez-Anaya (1996) reported. Moreover, the results of the wheat bread crumb sample which was studied, are in agreement with those reported by Hansen and Schieberle (2005). They reported that the number of volatiles is higher when sourdough is added, as in the case of compounds like 2-methyl-1-butanol, 3-methyl-1-butanol, 1-propanol, 2-phenylethanol, benzyl alcohol, 2-methylpropanoic acid, 3-methylbutanoic acid or acetic acid, all of which compounds shown in Table 4 are above $100\ \mu g\ K^{-1}$ (except $72.0\ \mu g\ K^{-1}$ of 3-methylbutanoic acid and $24.4\ \mu g\ K^{-1}$ of benzyl alcohol).

Regarding the second and third more abundant volatile compounds, 3-penten-2-ol and 4-vinylguaiacol, respectively, these display special concentration in the crumb. 4-vinylguaiacol is reported normally in crust (Rychlik & Grosch, 1996; Zehentbauer & Grosch, 1998) since it is generated in Maillard reactions through the decarboxylation of ferulic acid (Jiang, Chiaro, Maddali, Prabhu, & Peterson, 2009). It has also been reported in buckwheat bread (Lin et al., 2009) as it is present in great quantities in buckwheat flour (Damjan, Kantar, Kreft, & Prosen, 2009). In wheat bread crumb it has been found at trace level (Paraskevopoulou et al., 2012). Only Gassenmeier and Schieberle (1995) detected 4-vinylguaiacol as an important volatile compound in wheat bread crumb, prepared via pre-ferments (a mixture of flour, water and yeast that is fermented and added to the dough). In addition, Steensels et al. (2015) reported that the yeast species *Brettanomyces* and *Dekkera* are responsible for the generation of ethyl phenols, which are the reduced products of vinylphenols (Di Toro et al., 2015). The presence in sourdoughs of *Dekkera bruxellensis* (Meroth, Hammes, & Hertel, 2003) justifies then the generation of 4-vinylguaiacol in wheat bread crumbs when pre-ferments or sourdough have been employed. This is in line with commercial wheat bread including sourdough in the recipe. Finally, 3-penten-2-ol is an aroma compound that has never been reported in wheat bread crumb or crust. 1-penten-3-ol and 2-penten-1-ol have been barely reported in wheat breads (Birch, Petersen, Arneborg, et al., 2013; Birch et al., 2013). 1-penten-3-ol concentration increases in breads where enzymes have been added to sour-

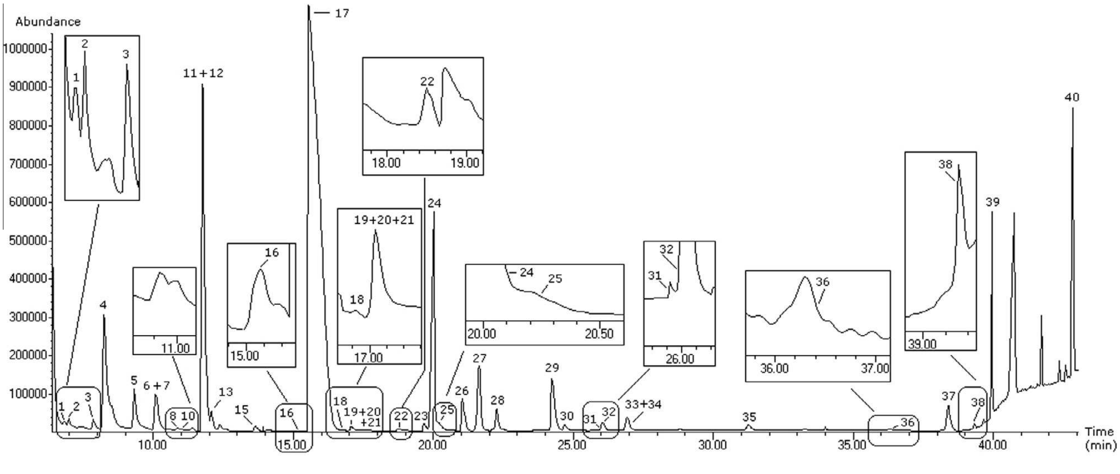

Fig. 2. Peaks obtained from the analysis of the commercial wheat bread crumb sample with the validated sample treatment. The numeration employed to name each peak is indicated in Table 1.

dough (Salim-ur-Rehman, Paterson, & Piggott, 2006), specifically in the form of soya flour (Luning, Roozen, Moëst, & Posthumus, 1991). However, as far as we know, it is the first time that 3-penten-2-ol has been reported in wheat bread, considering that is the second more abundant volatile in the analysed crumb sample. The large amount could be attributed to the use of baking enzymes in the bread recipe (sample description, Section 2.2), since concentration of the isomer 1-penten-3-ol is enhanced with the use of enzymes. Another isomer, 4-penten-2-ol, has been identified in triticale bread crumb (Sabovics, Straumite, & Galoburda, 2014), but triticale cereal is not classified as an ingredient on the commercial wheat bread label. The possible addition of sourdough to the recipe of the commercial bread analysed could also have an influence, since similar compounds like 3-nonen-1-ol and 2-nonen-1-ol have been encountered in wheat breads which contained sourdough (Plessas et al., 2008).

12 of the 38 volatile compounds detected in the wheat bread crumb sample analysed have been reported to correlate positively with the final aroma of bread: 3-methyl-1-butanol, 2-methyl-1butanol, benzyl alcohol, 2-nonenal (Salim-ur-Rehman et al., 2006), 2-phenylethanol (Hansen & Hansen, 1996), methional (Cho & Peterson, 2010), phenylacetaldehyde (Paraskevopoulou et al., 2012), furfural (Hansen & Hansen, 1996; Quílez, Ruiz, & Romero, 2006; Salim-ur-Rehman et al., 2006), 2,3-butanedione (Bianchi, Careri, Chiavaro, Musci, & Vittadini, 2008; Martínez-Anaya, 1996), acetoin (Quílez et al., 2006; Salim-ur-Rehman et al., 2006), 3-methylbutanoic acid (Salim-ur-Rehman et al., 2006) and 2-methylbutanoic acid (Grosch & Schieberle, 1997). On the other hand, only 5 aroma compounds have been reported to correlate in a negative way: hexanal (Martínez-Anaya, 1996), benzaldehyde, butyric acid, 2,4-(E,E)-decadienal (Quílez et al., 2006) and 1-octen-3-ol (Paraskevopoulou et al., 2012). Among those that correlate negatively, only butyric acid exceeds 100 μg K^{-1}. Therefore, the pleasant aroma of the commercial wheat bread crumb is justified.

4. Conclusions

An alternative method to analyse volatile compounds in wheat bread crumb has been proposed. The method consists of a Soxhlet extraction of the crumb with a diethyl ether/dichloromethane mixture which contains lipases. The extract was concentrated by means of a Vigreux column. The enzymes were responsible for hydrolysing the TAGs, DAGs and MAGs of the bread fat in order to obtain free fatty acids and glycerol. These fatty acids and glycerol could be volatilised due to the high temperatures of the final gradient of the GC, eluting in the final part of the chromatogram avoiding interferences. The method was validated, with high extraction efficiencies and good RSD% precision, and successfully applied to commercial wheat bread crumb analysis. The results agreed with those reported in the literature about wheat bread crumb, a wheat bread crumb characterised by alcohols, ketones, aldehydes, acids and small quantities of esters. There was neither creation nor elimination of volatile compounds between the sample obtained from Method A (without the addition of enzymes) and the sample obtained from the alternative Method B (with the use of lipases). The most abundant compounds were 3-hydroxy-2-butanone, 3-penten-2-ol, 4-vinylguaiacol, phenylethyl alcohol, acetic acid, 2-methylpropanoic acid, hexanoic acid and 2,3-butanediol. They have been commonly reported in bread crumb, with the exception of 4-vinylguaiacol, probably derived from the use of pre-ferments. It is the first time that 3-penten-2-ol is reported in the literature, which could have originated from the use of baking enzymes employed during bread elaboration. The least abundant compound was ethyl octanoate, in fact it was the only ester found. The small abundance of esters could be attributed to their losses during baking due to their high volatility.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.03.054.

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Analytical feasibility of a Solvent Assisted Flavour Evaporation (SAFE) method for aroma analyses in bread crumb

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Highlights

- ✓ Extraction efficiencies were better for the Lipases method, with an average of 87%.
- ✓ SAFE method showed matrix effect in lower number of volatiles than Lipases method.
- ✓ Intermediate precision was better in the Lipases method, always lower than 9%.
- ✓ Intra- and inter-day repeatability was better for the SAFE method, lower than 15%.
- ✓ Lipases method failed in analyses of breads with fat contents higher than 2%.

Abstract

The analysis of bread aroma is essential in order to evaluate its quality as well as to improve it. The use of different methodologies for the analysis of volatile compounds lead to varying results. In the present study, the matrix effect, extraction efficiency, limits of detection and quantification as well as the precision of a proposed solvent assisted flavour evaporation (SAFE) methodology were evaluated for the first time and compared with a reference method, both differing in the distillation step. The repeatability (lower than 8%) and matrix effect (present in 15 of the 31 compounds) were improved with the SAFE method but the extraction efficiencies (average of 52%) and the intermediate precision (higher than 15%) were not as requested. However, the applicability of the reference method was limited to breads with fat levels lower than 2%. For breads higher in fat, the SAFE method represents an alternative for aroma analysis.

Keywords: SAFE distillation; volatile compounds; wheat bread crumb; gas chromatography/mass spectrometry.

Abbreviations: AOAC (Association of Official Analytical Chemists); GC/MS (Gas chromatography/mass spectrometry); GC-O (Gas chromatography-olfactometry); LM (Lipases method); LOD (Limit of detection); LOQ (Limit of quantification); MSA (Method of standard addition); Q1 (Qualifier ion 1); Q2 (Qualifier ion 2); RSD (Relative standard deviation); SAFE (Solvent assisted flavour evaporation); SIM (Selected ion monitoring); SM (SAFE method); SPME (Solid phase microextraction); T (Target ion).

1. Introduction

Bread is one of the most commonly consumed foods around the world, and the quality of its aroma is decisive for its acceptance (Jensen, Oestdal, Skibsted, Larsen, & Thybo, 2011). The evaluation of the aroma of bread is essential to ensure this quality. In order to determine the volatile compounds that contribute to bread aroma, it is necessary to select a suitable analytical technique to isolate them from the bread matrix. Several options have been widely employed up to now, which can be divided into solvent extraction and headspace methodologies. Among the solvent extraction possibilities, vacuum sublimation (Sen, Laskawy, Schieberle, & Grosch, 1991), simultaneous steam distillation (Likens & Nickerson, 1964) and solvent assisted flavour evaporation (SAFE) (Engel, Bahr, & Schieberle, 1999) have been the most prevalently used. Meanwhile, as for the headspace options, the most widely utilised have been dynamic headspace (MacLeod & Ames, 1986) and solid phase microextraction (SPME) (Pawliszyn, 1997). Although the choice of the method depends on the type of food and the information being sought (Murat, Gourrat, Jerosch & Cayot, 2012), among a variety of other factors, SAFE and SPME have frequently been used in a complementary manner in order to obtain a complete profile of the overall flavour compounds (Corral, Salvador & Flores, 2015; Klensporf & Jeleń, 2008; Majcher & Jeleń, 2009; Thompson-Witrick et al., 2015; Wang, Song, Zhang, Tang & Yu, 2016). SAFE extracts have reportedly been richer in high-molecular weight volatile compounds, while SPME extracts have been richer in low-molecular weight volatile compounds (Mayuoni-kirshinbaum, Tietel, Porat, & Ulrich, 2012), which is very important for those compounds that are very volatile and co-elute with the solvent in the SAFE methodology (Majcher & Jeleń, 2009). Although many researchers select SPME because it is a quick, simple and solvent-free technique (Thompson-Witrick et al., 2015), SAFE has been reported to allow a more complete isolation, thereby more accurately preserving the native profile of the food product (Roth et al., 2014). Moreover, SAFE has been considered the best option for GC-O analysis due to its requirement of low extraction temperatures and presence of a solvent (Majcher & Jeleń, 2009). Therefore, SAFE has been recognised one of the main options in the analyses of volatile compounds in food.

In order to attain reliable results, analytical methods should be optimised and validated before being used. However, a limited number of studies have been conducted to examine the analytical characteristics of SAFE methodologies, such as intermediate precision (Havemose, Justesen, Bredie & Nielsen, 2007; Mo, Xu, & Fan, 2010; Thomsen, Gourrat, Thomas-Danguin, & Guichard, 2014), limits of detection and quantification (Majcher & Jeleń, 2009) or linearity (Pozo-Bayón, Guichard & Cayot, 2006) in different food matrices. The potential presence of matrix effect, the extraction efficiency percentages and the method repeatability have not been studied for SAFE. Moreover, to our knowledge, none of these analytical parameters have been examined in breads with the aim of validating the SAFE method. Only Pozo-Bayón et al. (2006) studied the linearity of a SAFE method through the quantification of volatile compounds in wheat breads using the method of standard addition (MSA). However, the purpose of Pozo-Bayón (2006) study was to evaluate the percentage of aroma compounds retained in bread after processing of flavoured doughs, percentage of retention that included not only the SAFE extraction efficiency but also the losses of aroma compounds during baking.

The aim of the present work was to evaluate the analytical characteristics of the proposed SAFE method (SM) for the analysis of 31 main volatile compounds in wheat bread crumb. For this purpose, the recovery percentages, matrix effects and extraction efficiencies, the limits of detection (LODs) and quantification (LOQs) as well as the precision (intra- and inter-day repeatability and intermediate precision) were examined. In order to evaluate the SAFE distillation step, the proposed method was compared to the validated solvent extraction methodology reported previously by our research group (Lipases method, LM) (Pico, Nozal, Gómez, & Bernal, 2016).

2. Materials and methods

2.1. Materials, reagents and standards

Dichloromethane and acetone were purchased from LAB-SCAN (Gliwice, Poland) and diethyl ether was acquired from Panreac (Barcelona, Spain). Liquid nitrogen was obtained from Carburos Metálicos (Barcelona, Spain) and dry ice was purchased from Linde Group (Munich, Germany). The enzyme Lipozyme CALB L® (from *Aspergillus niger*) was kindly provided from Novozymes (Bagsvaerd, Denmark). The standards (Sigma-Aldrich, Steinheim, Germany) were all pure and they are listed in Table 1.

2.2. Preparation of standard solutions

Stock solutions of each volatile compound were prepared in acetone in a concentration of 10 g L^{-1} (Table 1). The working solutions were prepared from the mix of stock solutions as required. All the solutions were stored in a freezer at -21 °C.

2.3. Sample description and sampling

The development and characterisation of the SM as well as comparison with the LM were all carried out with the crumb of wheat bread purchased from Forva (Puçol, Spain). The label indicated that the ingredients were wheat flour, water, salt, yeast and flour improver (wheat flour, anti-caking agent (E-170), emulsifier (E-472e), antioxidant (E-300) and enzymes).

The loaf of bread was cut into slices of 5 cm width and the ends were discarded. The crumb was carefully separated from the crust with a distance from crumb to crust of 1 cm. Then the crumb was frozen with liquid nitrogen and ground in an Ika grinder model M20 (Staufen, Germany) for 10 seconds.

Fat in breads can come mainly from the flour, although additional fat is added when oil included in the recipe. In order to check the maximum amount of fat supported by the LM, flours with different fat content were submitted to Soxhlet extraction. Maize starch (< 0.5% fat) was purchased from Unilever (Barcelona, Spain), rice flour (1.2% fat) from Sarchio (Carpi, Italy), teff flour (2.1% fat) from Saluteff (San Martín del Valle, Spain), buckwheat flour (3.1% fat) from Nature & Cie (Vallet, France) and quinoa (6.0% fat) from Anapqui (La Paz, Bolivia).

2.4. Sample treatments

2.4.1. SAFE method

Figure 1 represents the scheme of the proposed SAFE methodology. It consists mainly of a Soxhlet extraction followed by a SAFE distillation and a final concentration by means of a Vigreux column. First, 50 g of the frozen bread crumb powder was inserted into the Soxhlet thimble and introduced in the Soxhlet apparatus. The extraction was carried out with 300 mL of a mixture of diethyl ether / dichloromethane (2:1) for 5 h at 40 °C and then the distillation flask was cooled to room temperature for 10 min. Afterwards the extract was concentrated by means of a 50 cm Vigreux column for 20 min (until approximately 150 mL) and finally left to equilibrate to room temperature for another 10 min. Then, the extract was distilled employing a SAFE apparatus (Baeng, Manching, Germany), in order to separate the volatile compounds and solvent from the fat and other non-volatile molecules (Engel et al., 1999). The distillation flask, the central head and the legs were thermostated at 21 °C using a water recirculation thermostat (Huber, New Jersey, USA). A vacuum of 10^{-6} mbar was achieved by means of a HP 40 B2 from Vacuubrand (Wertheim, Germany). The cooling trap was filled with dry ice and the condensation flask was also immersed in a Dewar vessel full of dry ice in order to condense the sample but not completely freeze it (as opposed to the case when using liquid nitrogen). The extract was added by dropping 10 mL of sample every two minutes from the dropping funnel. As the distilled extract immersed in the Dewar vessel was very cold, 20 min were needed to allow it to reach room temperature. Then, the extract was concentrated again using the Vigreux column until a final volume of 1 mL was achieved, which required 60 - 65 min at 38 °C.

Finally, the extract was left again for 10 min to achieve room temperature. The sample was immediately injected into GC/MS. A careful cleaning of the SAFE system was necessary after each sample analysis, as otherwise contamination phenomena were observed.

2.4.2. Lipases method

The procedure was reported by Pico et al. (2016): 50 g of the frozen bread crumb powder were extracted by employing the Soxhlet system with 300 mL of a mixture of diethyl ether / dichloromethane (2:1) that contained 25 µL of the lipase enzyme Lipozyme CALB L®. The extraction was carried out for 5 h at 40 °C. As the lipases were added into the solvent, the fat extracted with the solvent was hydrolysed by the lipases at the same time that the solvent extraction was occurring. Following extraction, the distillation flask was cooled to room temperature for 10 min. The extract was then concentrated to a volume of 1 mL with a 50 cm Vigreux column, which required approximately 90 min at a temperatura of 38 °C. Finally, the extract was left again for 10 min to achieve room temperature. The sample was immediately injected into GC/MS.

2.5. GC/MS instrumentation and chromatographic conditions

GC/MS analyses were performed on a 7890A Gas Chromatograph (GC) coupled to a 5975C Mass Spectrometer (MS) detector, all from Hewlett Packard (Palo Alto, California, USA). Separation was achieved on a polar ZB-Wax column (100% polyethylene glycol, 60m × 0.25mm ID × 0.25µm) obtained from Phenomenex (Torrance, California, USA). The GC was operated under programmed temperature conditions ranging from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C /min, after which the temperature was increased to 114 °C (3 min) at 3.5 °C /min, and then to 136 °C (0 min) at 2.5 °C /min. Finally, the temperature was raised to 245 °C at 85 °C /min (25 min). The total run time was 52 min. An injection volume of 1 µL was employed by the autosampler in pulsed splitless mode. The inlet temperature was set at 250 °C and the carrier gas was Helium supplied by Carburos Metálicos (Barcelona, Spain) with a flow rate of 1.1 mL/min. The interface, ion source and quadrupole temperatures were 250 °C, 230 °C and 150 °C, respectively. The MS operated in positive electron impact mode with an ionization energy of 70 eV. Analyses were performed with selected ion monitoring mode (SIM), with one target (T) and with three qualifier ions (Q_1, Q_2, Q_+) for each volatile compound (see Table 1). All the analytes were identified and confirmed by a comparison of their retention times and mass spectra with standards and the Mass Spectra Library (Wiley 7N edition).

2.6. Analytical characterisation of the SAFE method (SM)

The analytical parameters were evaluated following the AOAC guidelines (2002).

2.6.1. Recovery percentages, matrix effect and extraction efficiency

2.6.1.1. Recovery percentages

50 g of frozen crumb powder was spiked with 10 mL of 2.4 mg L^{-1} standard mixture. Next, both the spiked sample and the blank (to subtract the signal) were treated simultaneously. The spiked sample and the blank were prepared in duplicate and injected consecutively in triplicate (n=6). A standard mixture of 24 mg L^{-1} injected in triplicate (n=3) was employed to calculate the recovery percentages.

2.6.1.2. Matrix effect

The extract obtained from the Soxhlet extraction and concentrated below 1 mL with the Vigreux column, was spiked with 100 µL of a standard mixture of 240 mg L^{-1} and finally made up to the volume. The blank underwent the sample treatment simultaneously. Both samples were prepared in duplicate and injected in triplicate (n=6). A standard mixture of 24 mg L^{-1} injected in triplicate (n=3) was employed to calculate the recovery percentages.

2.6.1.3. Extraction efficiency

This parameter was evaluated by comparing the recovery percentages (which take into account the extraction efficiencies and the matrix effect) with the matrix effect values (which only take the effect of the MS detector into consideration). It evaluates the authentic yield of the sample treatment and it is considered one of the main parameters evaluated for the optimisation of a method. It should be between 75% and 120% for concentrations around 1 µg g^{-1} (AOAC guidelines, 2002).

2.6.2. Limits of detection (LODs) and quantification (LOQs)

These parameters were calculated by considering the area of analyte peaks from a spiked sample (480 µg K^{-1}) and the area of the noise from a solvent blank at the same retention time as that of the analyte peaks. Injections were made in quintuplet (n=5). The solvent used to measure the noise was a mixture of diethyl ether/dichloromethane (2:1).

2.6.3. Precision: intra-day repeatability, inter-day repeatability and intermediate precision

For intra-day repeatability, an extract of a spiked sample (480 µg K^{-1}) was injected in quintuplet and the RSD (%) of each compound was calculated (n=5). In terms of inter-day repeatability, the same extract of a spiked sample was injected in triplicate on three alternate days and RSD (%) was calculated (n=9). Finally, to study the intermediate precision, a spiked sample was prepared in duplicate by different analysts, as in section 2.4.1, and injected in triplicate for calculate the RSD (%) (n=6).

Following the AOAC guidelines (2002), for concentrations of 1 µg g^{-1}, maximum RSDs of 11% are accepted for the intermediate precision and of 8% for the repeatability.

3. Results and discussion

3.1. Selection of the SAFE method conditions for bread crumb aroma analyses

In order to achieve an optimal distillation temperature, 10 mL of a 24 mg L^{-1} standard mixture in dichloromethane was tested in the SAFE system at eight different temperatures: 21 °C, 23 °C, 25 °C, 26 °C, 27 °C, 28 °C, 29 °C and 30 °C. The best recovery percentages for the whole range of volatile compounds were obtained at 26 °C, for almost all the volatile compounds had nearly 100% (data not shown). However, when an extract of bread crumb was tested at 26 °C, the 10 mL of sample started to distil almost immediately and vigorously, probably due to the presence of the fat. This turbulent distillation could lead to losses of volatile compounds, and problems with the SAFE system due to the fragility of the glass. Thus, lower temperatures of 21 °C and 23 °C were tested with the bread sample. When the temperature rose from 21 °C to 23 °C, there was a moderate reduction of the signal for 19 of the 30 compounds while 10 compounds kept their signals approximately constant (data not shown). Only the peak areas of 2,4-decadienal and 4-vinylguaiacol were slightly higher at 23 °C, since they present low boiling points. Therefore, 21 °C was selected as the optimal temperature for distilling wheat bread crumb extracts in the SAFE system. The Soxhlet and Vigreux conditions were kept identical to the LM, since the method was successfully validated (Pico et al., 2016).

3.2. Analytical characterisation of the SAFE method

3.2.1. Extraction efficiencies of the SAFE method compared to the Lipases method

3.2.1.1. Recovery percentages and matrix effect

The excessively high recovery percentages for acetic acid and acetoin in the SM as well as the extremely low recovery percentages for 1-pentanol, ethyl hexanote, R-(+)-limonene, methional, 1-octen-3-ol, phenylacetaldehyde, 1,3-butanediol, benzyl alcohol, 2,4-decadienal and 4-vinylguaiacol, led to the possible presence of matrix effects that increased or decreased the signal during ionisation. Effectively, for 11 of the 31 volatile compounds there was a considerable matrix effect of suppression of ionisation; moreover, there was a great increase in ionisation (Table 2) only for acetoin, acetic acid, 2,3-butanediol and butyric acid. Finally, because the % of matrix effect was lower than 10% for the other 16 volatile compounds, no matrix effect was considered for them. In the case of the LM, 13 volatile compounds did not present a matrix effect, but 10 of them showed an increase in the ionisation and 8 of them a decrease in signal (Table 2). Therefore, the SM exhibited matrix effect in a lower number of volatile compouds than LM, which could be attributed to the physical removal of the non-volatile compounds during SAFE distillation. However, both for SM and LM, the use of MSA would be necessary for quantification of the volatile compounds (Pozo-Bayón et al., 2006). Thus, the application of MSA would involve the study of the linearity.

3.2.1.2. Extraction efficiencies

By adjusting the recovery percentages with the matrix effects, extraction efficiencies were obtained. For SM, these extraction efficiencies were between 6.2% and 148.6%, while for LM they were between 55.5% and 116.9%. As depicted in Figure 2, all the chemical groups experienced a great decrease in extraction efficiencies with the SM. The differences between LM and SM for the acids were smaller, merely 7%, because they presented the highest extraction efficiencies for the SM. This could be attributed to good SAFE distillations and similar interactions between the acids and the amylose of the crumb during Soxhlet extraction (Le Bail, Biais, Pozo-Bayón & Cayot, 2004).

In the case of LM, 22 volatile compounds yielded extraction efficiencies higher than 75%. Five of them were around 70% and the last 4 were near to 60%, with an average extraction efficiency of 87.1%. However, in the case of SM the results were not suitable, even though the SM was optimised with a standard mixture and it only differed from LM by its SAFE distillation step. It was probably due to the lost of the volatile compounds when handling the sample in the SAFE apparatus. For SM, only 3-penten-2-ol, 1-hexanol and isobutyric acid presented extraction efficiencies higher than 75%, while the other 28 volatile compounds had extraction efficiencies ranging between 6.2 and 69.6%. In fact, the average extraction efficiency was 51.9%, which is far from an optimum value. The extraction efficiencies of R-(+)-limonene, methional and benzaldehyde were similar in both methods. 2,3-Butanediol and 1,3-butanediol exhibited the worst extraction efficiencies for SM, with levels around 6%. The diols are highly polar compounds that were probably lost due to adsorption in any glass material while the sample was being handled. Nevertheless, low extraction efficiencies for SM have been reported in general by Engel et al. (1999): notably, when distilling a mixture of 7 aroma compounds dissolved in a matrix with 50% fat, extraction efficiencies ranged between 0.5% and 37%. As most flavour compounds are hydrophobic, fat content should highly influence flavour extraction (Pozo-Bayón et al., 2006). Although the fat content in the commercial bread used in the present study was 1.1%, which is much lower than 50%, Engel et al. (1999) analysed the volatile compounds in a synthetic matrix of tri-acylglycerides without di- and mono-acylglycerides, which would have undoubtedly affected the distillation due to the retention of volatile compounds. Engel et al. (1999) reported yields of 37% for 3-methylbutanoic acid, 26% for phenylacetaldehyde, 13% for phenylethanol and 4% for 2,4-decadienal. In the present study, the extraction efficiencies were 56%, 31%, 60% and 23% for the same compounds, respectively. The better results are likely to be due to the lower content in fat.

To our knowledge, there is no data regarding the extraction efficiencies of SAFE methodologies in bread. The suitability of the SAFE method has normally been tested with the total amount of volatile compounds extracted compared with other extraction methods (Kashima & Miyazawa, 2014; Murat et al., 2012). However, high amounts of volatile compounds do not ensure good extraction efficiencies. There could be a very good extraction only for a few compounds and really poor for the rest, compensating the total amount of volatile compounds extracted. Moreover, the importance in the final aroma of bread should be taken into consideration in the total amount of volatile compounds extracted.

3.2.2. LODs and LOQs of the SAFE method compared to the Lipases method

For the SM, the LODs ranged between 0.2 and 54.9 µg K^{-1} (5.4 µg K^{-1} average), while the LOQs were in the range of 0.5 and 182.9 µg K^{-1} (18.1 µg K^{-1} average) (Table 3).

For the LM the results were similar, with LODs ranged between 0.3 and 33.2 µg K^{-1} (3.3 µg K^{-1} average) and LOQs in the range of 1.0 and 110.8 µg K^{-1} (11 µg K^{-1} average) (Table 3). As the order of magnitude of the LODs and LOQs for SM and LM is the same, the physical removal of the matrix in SM is not expected to strongly affect the signal-to-noise relationship.

Majcher & Jeleń (2009) found LODs values (0.8-89 µg K^{-1}) of the same order of magnitude when they employed a SAFE method for the analysis of volatile compounds in extruded potato snacks. Although they used the same SAFE apparatus, the values slightly differed because the matrix was not the same and also, above all, because they utilised different methodology (steps, extractant, sample amount, extraction time, etc).

3.2.3. Precision of the SAFE method (SM) compared to the Lipases method (LM)

3.2.3.1. Intermediate precision

The intermediate precision, expressed as % RSD, for SM varied between 3.9% and 26%, and for LM it varied between 0.1% and 8.9% (Table 3). The average of the intermédiate precision of the 31 volatile compounds for SM and LM was 15.3% and 4.6%, respectively. This parameter reflects the relative standard deviation among replicates of the same sample prepared on different days or by different analysts. Therefore, the more tedious the sample treatment, the more difficult the replication of the method and the higher the RSD. The SM mainly consisted of three steps (Soxhlet extraction, SAFE distillation and Vigreux concentration), while the LM consisted of only two steps (Soxhlet extraction containing lipases and Vigreux concentration). The SAFE distillation of the extract resulting from the Soxhlet extraction required approximately 1h, taking into consideration the distillation itself and the thawing of the distillate. Moreover, the extract resulting from the Soxhlet extraction was added 10 mL each time (Engel et al., 1999), which could promote the losses of volatile compounds, justifying the high % RSDs for the intermediate precision of SM. For the SM, 27 of the 31 volatile compounds presented RSDs higher than 11%, while for the LM all the volatile compounds showed RSDs lower than 9%.

Despite the high RSDs of our SM, high % RSDs for intermediate precision have been commonly reported in the literature for SAFE methods in several matrices (yet, to our knowledge, no existing data has been reported for bread). Majcher & Jeleń (2009) found RSDs of intermediate precision around 14% for their SAFE method, although they replicated the preparation of a standard instead of a sample. Furthermore, Mo et al. (2010) reported that not all the RSDs for their three replicates of Chinese rice wine were satisfied, due to the tedious SAFE procedure. Havemose et al. (2007) reported % RSDs between 20% and 95% for a SAFE method analysing the volatile compounds in milk, although for 20 of the 27 volatile compounds the RSDs were below 40%. Average RSDs of 45% were also reported by Thomsen et al. (2014) for a SAFE methodology for volatile compounds in cheese.

3.2.3.2. Intra-day repeatability and inter-day repeatability

For SM, the intra-day repeatability values were lower than 3% for all the volatile compounds, while the inter-day repeatability values for 27 volatile compounds were lower than 8% and, for the other 4 compounds, they were below 15%. In the case of LM, only one volatile compound had an intra-day repeatability higher than 8% (RSD 9.7%). Thus the intra-day repeatability was acceptable for both methods (Table 3). However, for the inter-day repeatability of the LM, there were 16 volatile compounds with RSDs lower than 8% but the other 15 compounds were between 8% and 14.8%. Therefore, SM presented better RSDs for the inter-day repeatability than LM (Table 3), probably due to the physical removal of the non-volatile compounds from the matrix. Nevertheless, the AOAC validation guidelines allow RSDs of up to 15% for repeatability for concentrations of 1 µg K^{-1}, and our samples were spiked between 1 µg g^{-1} and 1 µg K^{-1}. Moreover, the results for the inter-day repeatability for the LM only imply that the extract should be injected on the same day as the analysis, something advisable regardless of the RSDs.

3.3. Applicability of the SAFE method for bread aroma analyses

Each extraction method has advantages and drawbacks depending on the food matrix studied (Murat et al., 2012). Considering the matrix effect and the intra- and inter-day repeatability, the proposed SM presented slightly better results than the reported LM, probably due to the physical removal of the non-volatile compounds from the extract resulting from the Soxhlet extraction. The issue regarding the presence of a small amount of non-volatile molecules in the LM was mitigated with the backflush function of the GC/MS and could be improved with the use of a GC pre-column. Moreover, both SM and LM offered similar LODs. However, it is well known that the extraction efficiency and the intermediate precision are key parameters for method optimisation and these were much better for the LM. In fact, both parameters in the SM did not meet the requirements of the validation guidelines of the AOAC. On the other hand, the inclusion of the SAFE distillation led to nearly 1h of additional sample treatment time without taking into consideration the careful cleaning of the SAFE apparatus between samples to avoid contamination. Therefore, the LM is suggested for the analysis of volatile compounds in wheat bread crumb.

However, when the LM was applied to crumbs of breads with higher fat content than wheat, the final Vigreux concentration to 1 mL was not possible because the extract was mainly dissolved fat. The LM was applied to a wide range of breads with different fat contents (data not shown), including breads prepared with maize starch (< 0.5%), wheat (1.1%), rice (1.2%), teff (2.1%), buckwheat (3.1%) and quinoa (6.0%). The Soxhlet extract from teff bread crumb could not be reduced to less than 2 mL with the Vigreux concentration, the extract was mainly fat. Therefore, the LM is limited to breads with fat contents lower than 2%. As a result, the SM could be a good option for breads with fat contents exceeding 2% since the fat is physically removed, in spite of the limitations regarding the extraction efficiencies and the intermediate precision. By all means, the analytical characteristics of the SAFE method should be evaluated before applying it to a new matrix in order to ensure reliable results.

4. Conclusions

Upon examining the main analytical parameters (matrix effect, extraction efficiencies LOD/LOQ and precision) of the proposed SAFE method (SM), it was found that the matrix effect and the repeatability slightly improved for SM compared to the Lipases method (LM), as a consequence of the total removal of non-volatile compounds. Nevertheless, MSA would be necessary in case of quantification of the volatile compounds for both methods. On the other hand, the SM presented unsuitable extraction efficiencies and its RSDs for intermediate precision were higher than those recommended by AOAC guidelines, in concordance with reported literature. Thus, the LM seemed to be more suitable for the analysis of volatile compounds in wheat bread. Nonetheless, LM was limited to breads with fat contents lower than 2% due to its failure during the Vigreux concentration.

Therefore, for breads with fat contents higher than 2%, the SM represents a good alternative. For aroma analyses of wheat bread, most of the extraction efficiencies of the SM were near 50%, thereby enabling qualitative-comparative analyses. Moreover, the use of an internal standard could improve the intermediate precision, thus reducing the RSDs. By all means, it is highly advisable to examine the analytical characteristics of a SM if aroma analyses of breads with high content of fat are made, even at qualitative levels.

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Table 1. Volatile compounds studied in wheat bread crumb, in order of elution (Rt, retention time). Target (T) and qualifier (Q1, Q2, +Q) ions were employed for each compound.

Volatile compounds	Rt	T	Q1	Q2	Q+
Hexanal	8.933	56	44	72	82
3-Penten-2-ol	9.263	71	43	53	86
2-Methyl-1-butanol	11.247	55	70	41	57
3-Methyl-1-butanol	11.251	57	41	70	29
1-Pentanol	11.948	42	55	70	91
Acetoin	12.294	45	88	27	15
Ethyl hexanoate	12.981	88	99	43	60
R-(+)-Limonene	13.649	68	93	79	107
1-Hexanol	14.197	56	41	42	55
Acetic acid	14.541	45	60	15	29
Furfural	16.507	96	39	29	67
Methional	16.781	48	104	76	61
1-Octen-3-ol	17.824	57	72	43	85
2,3-butanediol	18.11	45	57	29	75
Isobutyric acid	18.928	43	41	73	27
Benzaldehyde	19.711	106	105	77	51
Ethyl octanoate	19.066	88	101	127	57
5-Methyl-2-furaldehyde	20.775	110	109	53	81
Butyric acid	21.164	60	73	42	27
Furfuryl alcohol	22.198	98	81	69	53
Butyrolactone	22.041	42	28	86	56
2-(E)-Nonenal	22.182	70	55	41	83
3-Methylbutanoic acid	22.919	60	43	87	39
2-Methylbutanoic acid	22.940	57	74	87	41
Phenylacetaldehyde	23.683	91	120	92	65
1,3-Butanediol	25.392	43	45	57	72
Hexanoic acid	27.101	60	73	87	41
Benzyl alcohol	27.374	79	108	91	51
Phenylethyl alcohol	28.020	91	122	65	77
2,4-(E,E)-Decadienal	28.603	81	67	95	152
4-Vinylguaiacol	30.372	150	135	107	77

Table 2. Recovery %, matrix effect and extraction efficiencies of the SAFE method (SM) compared to the Lipases method (LM).

Volatile compounds	**% Recovery**		**% Matrix effect**		**% Extraction efficiency**	
	SM	**LM**	**SM**	**LM**	**SM**	**LM**
Hexanal	33.1	70.1	89.5	53.2	43.6	116.9
3-Penten-2-ol	72.4	121.9	87.1	117.2	85.3	104.7
3-Methyl-1-butanol	63.9	80.9	114.0	112.9	49.9	67.9
2-Methyl-1-butanol	51.0	111.0	101.0	119.8	50.0	91.2
1-Pentanol	5.8	48.2	55.8	37.3	50.0	110.8
Acetoin	111.7	142.7	142.1	142.3	69.6	100.5
Ethyl hexanoate	31.5	70.8	79.1	98.6	52.4	72.1
R-(+)-Limonene	24.1	58.0	61.1	94.7	63.0	63.4
1-Hexanol	72.2	44.7	75.9	43.5	96.2	101.2
Acetic acid	210.7	88.5	162.1	87.9	148.6	100.6
Furfural	43.7	93.8	95.2	105.8	48.4	88.1
Methional	23.9	56.5	71.8	93.4	52.1	63.2
1-Octen-3-ol	37.5	75.8	78.4	89.5	59.0	86.3
2,3-butanediol	93.8	96.5	187.6	99.3	6.2	97.2
Isobutyric acid	87.2	87.8	102.4	78.9	84.8	108.9
Benzaldehyde	45.0	55.0	93.5	99.4	51.5	55.5
Ethyl octanoate	47.5	122.9	95.9	111.5	51.6	111.4
5-Methyl-2-furaldehyde	46.2	76.5	92.5	103.8	53.8	72.7

Table 2. (continued)

Volatile compounds	% Recovery		% Matrix effect		% Extraction efficiency	
	SM	**LM**	**SM**	**LM**	**SM**	**LM**
Butyric acid	92.4	78.0	133.2	104.9	59.1	73.1
Furfuryl alcohol	46.9	116.8	97.2	101.1	49.7	115.7
Butyrolactone	48.8	88.0	107.3	73.3	41.5	114.7
2-(E)-Nonenal	32.9	104.0	93.1	107.1	39.8	96.9
3-Methylbutanoic acid	59.2	96.0	103.3	120.8	55.9	75.2
2-Methylbutanoic acid	69.3	107.6	102.0	126.5	67.3	81.1
Phenylacetaldehyde	19.4	79.0	88.9	85.8	30.5	93.2
1,3-Butanediol	13.6	99.2	107.0	105.0	6.6	94.2
Hexanoic acid	64.5	103.9	115.3	134.6	49.3	69.3
Benzyl alcohol	32.4	89.4	98.8	112.8	33.5	76.6
Phenylethyl alcohol	70.9	96.3	111.2	100.1	59.7	96.2
2,4-(E,E)-Decadienal	6.5	70.3	83.4	107.4	23.0	62.9
4-Vinylguaiacol	0.1	98.2	54.9	121.5	45.1	76.6

Table 3. LODs, LOQs and precision parameters of the SAFE method (SM) compared to the Lipases method (LM).

Volatile compounds	LOD		LOQ		Intermediate precision % RSD		Intra-day repeatability % RSD		Inter-day repeatability % RSD	
	SM	LM	SM	LM	SM	LM	SM	LM	SM	LM
Hexanal	5.5	2.6	18.5	8.7	14.1	6.6	0.6	0.0	3.1	5.0
3-Penten-2-ol	0.6	0.4	2.1	1.2	13.0	5.6	0.2	5.8	8.7	5.2
3-Methyl-1-butanol	2.5	1.4	8.3	4.8	16.1	1.6	0.1	6.2	3.6	7.9
2-Methyl-1-butanol	1.4	0.9	4.7	3.0	18.4	6.6	0.1	0.1	5.2	5.6
1-Pentanol	13.0	7.8	43.4	26.0	18.0	5.8	0.7	9.7	15.2	14.8
Acetoin	0.6	0.5	1.9	1.6	12.7	2.2	0.1	6.2	4.3	6.9
Ethyl hexanoate	1.9	0.8	6.2	2.8	17.7	7.8	0.3	5.9	3.9	6.9
R-(+)-Limonene	4.3	0.8	14.4	2.8	15.5	4.1	0.1	6.1	2.8	7.6
1-Hexanol	0.4	0.7	1.4	2.3	3.9	7.3	0.2	6.2	3.4	8.7
Acetic acid	0.2	4.6	0.5	15.2	26.0	8.0	0.2	1.6	1.8	5.3
Furfural	2.2	1.0	7.4	3.4	19.4	0.6	0.2	5.0	6.8	7.2
Methional	25.1	1.5	83.7	5.2	9.3	7.6	0.2	4.6	3.4	7.2
1-Octen-3-ol	1.0	2.0	3.3	6.5	18.1	1.6	0.3	6.8	5.7	8.1
2,3-butanediol	1.0	2.2	3.2	7.4	15.3	3.7	0.4	4.7	3.5	8.8
Isobutyric acid	2.1	2.1	7.0	7.0	15.3	1.0	0.4	4.1	1.7	11.4
Benzaldehyde	1.1	0.9	3.6	3.0	19.9	3.7	1.1	5.1	4.0	7.7
Ethyl octanoate	1.9	0.3	6.4	1.0	17.8	5.0	0.2	5.3	4.9	13.7
5-Methyl-2-furaldehyde	1.9	3.4	6.4	11.3	19.4	4.2	1.2	4.5	3.4	7.4
Butyric acid	1.9	2.3	6.4	7.5	19.5	2.3	0.1	4.5	3.3	9.6
Furfuryl alcohol	2.1	2.0	6.9	6.7	16.1	8.5	1.3	4.6	4.8	8.6

Table 3 (continued)

Volatile compounds	LOD		LOQ		Intermediate precision % RSD		Intra-day repeatability % RSD		Inter-day repeatability % RSD	
	SM	**LM**	**SM**	**LM**	**SM**	**LM**	**SM**	**LM**	**SM**	**LM**
Butyrolactone	2.2	1.2	7.2	4.0	11.2	7.5	0.2	4.0	8.1	8.4
2-(E)-Nonenal	4.0	2.4	13.2	7.9	5.7	5.4	0.4	4.4	7.7	8.7
3-Methylbutanoic acid	1.1	0.7	3.7	2.3	19.9	0.9	2.4	4.5	5.4	9.1
2-Methylbutanoic acid	3.0	1.9	10.0	6.4	20.5	2.6	0.7	4.0	9.2	8.9
Phenylacetaldehyde	2.1	1.2	7.0	4.2	12.5	7.3	1.4	1.0	4.8	11.2
1,3-Butanediol	54.9	9.9	182.9	32.9	12.7	2.7	1.8	3.1	6.1	4.2
Hexanoic acid	5.2	3.2	17.4	10.8	14.0	8.7	2.6	4.2	3.9	8.5
Benzyl alcohol	2.2	4.2	7.4	14.1	12.0	2.1	0.9	7.0	7.6	9.7
Phenylethyl alcohol	4.6	3.4	15.4	11.4	10.8	3.4	0.5	4.7	7.4	6.7
2,4-(E,E)-Decadienal	12.5	33.2	41.6	110.8	12.0	5.0	1.1	0.4	4.3	8.7
4-Vinylguaiacol	nd	3.0	nd	10.1	16.6	3.2	1.9	5.0	11.7	5.5

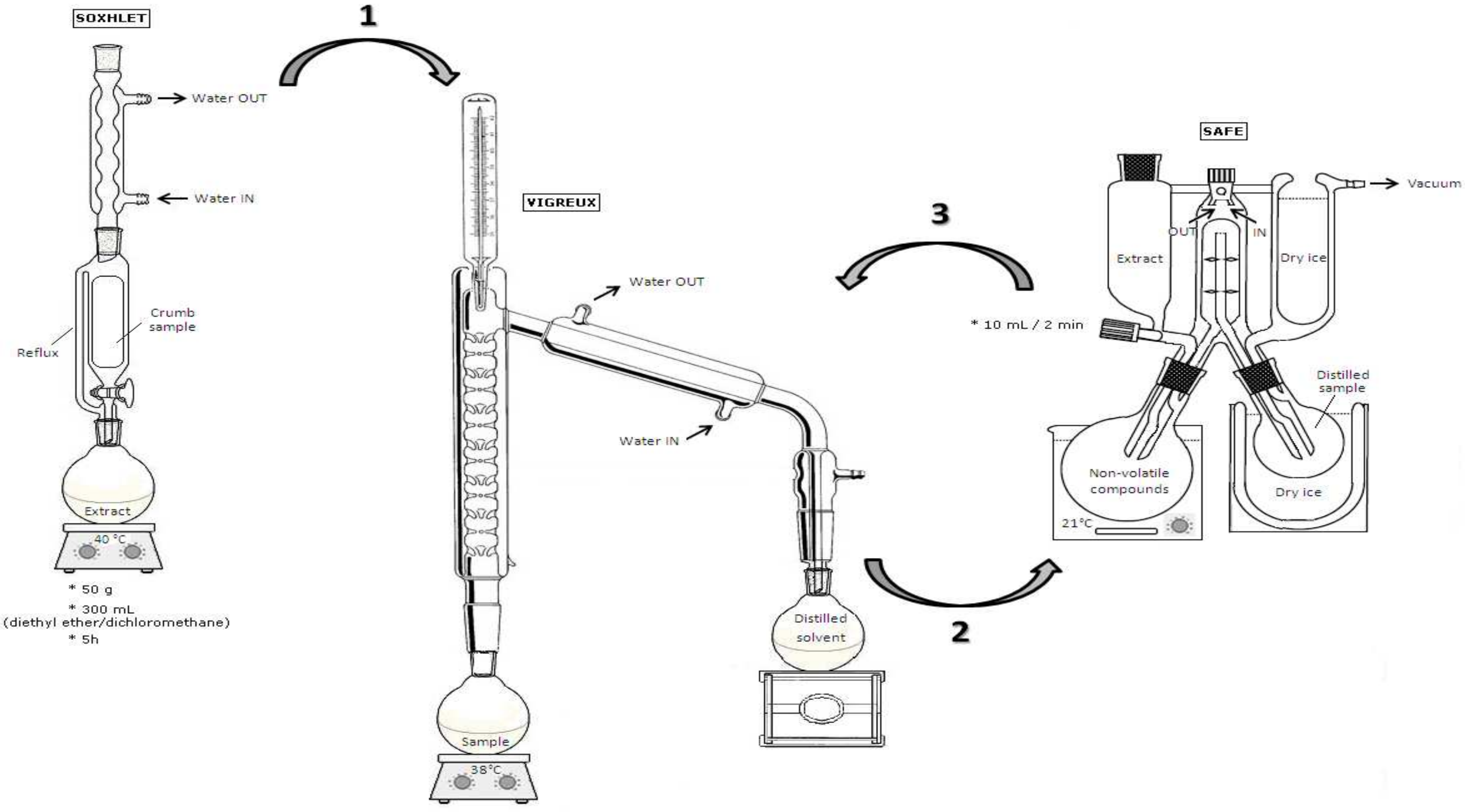

Fig.1. Scheme of the SAFE method (SM) proposed for the analysis of volatile compounds in wheat bread crumb. Soxhlet extraction (first step), Vigreux concentration (second step), SAFE distillation (third step) and final Vigreux concentration (fourth step).

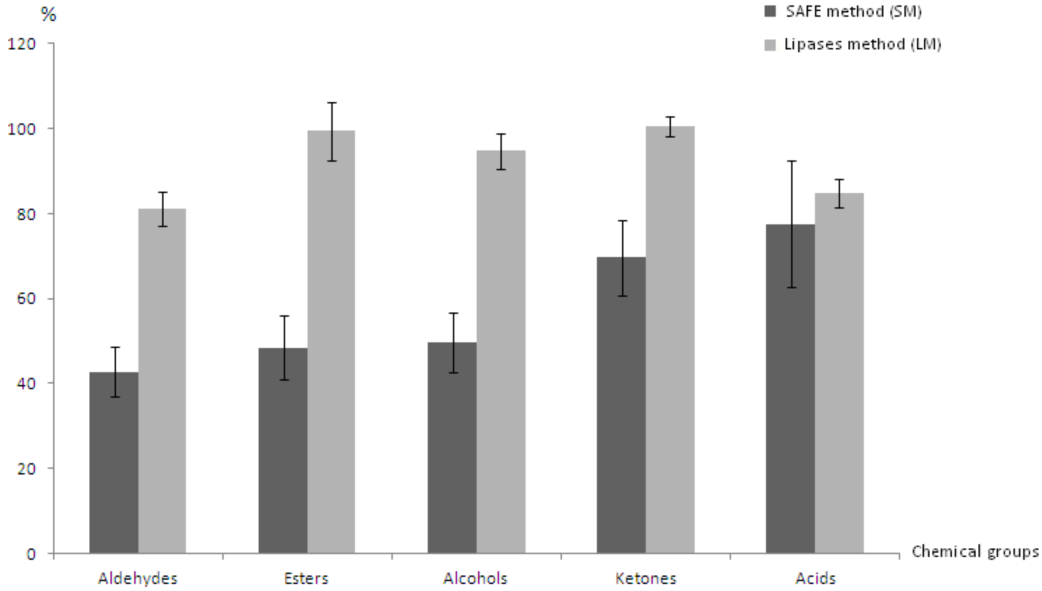

Fig.2. Average extraction efficiencies, in %, obtained from the SAFE method (SM) and the Lipases method (LM) for the main chemical groups of volatile compounds in wheat bread crumb.

VI. Section 2

Approaches for achieving accurate analyses of volatile compounds in bread doughs and crumbs

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Impact of frozen storage time on the volatile profile of wheat bread crumb

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ABSTRACT

The freezing of wheat bread before aroma analyses is a common practice in order to preserve loss of the volatile profile. However, the impact of the frozen storage time on the aroma profile has not been studied. For this purpose, the volatile profiles of wheat bread frozen for 1, 2 and 4 weeks were analysed employing solvent extraction and static headspace methodologies with GC/MS. The results revealed that the freezing was effective to prevent the loss of volatiles during the first week. However, after two weeks, there was an increase of volatile compounds, probably generated by chemical reactions. Thus, a maximum of one week of frozen storage was recommended when using the solvent extraction methodology. When using the static headspace method, the samples should be analysed on the same day as preparation, since the extraction was surprisingly increased due to the starch retrogradation that occurred during freezing.

1. Introduction

The aroma of bread is one of the main characteristics perceived by consumers. The more attractive the aroma is, the more likely the bread will be consumed. Thus, the development of new recipes that improve the bread aroma as well as the quality control of the bread aroma itself are key factors to ensure consumer acceptability. Therefore, accurate analytical methods are essential to measure the aroma of bread. In this context, the freezing of wheat bread samples, in order to preserve the volatile compounds, is usually required prior to chemical analyses due to logistic questions of shipping or production on a different day than the analyses. Numerous studies reported the "freezing of the bread sample until the aroma analysis" without checking if the volatile profile even changed at freezing temperatures (Bianchi, Careri, Chiavaro, Musci, & Vittadini, 2008; Luning, Roozen, Moëst, & Posthumus, 1991; Paraskevopoulou, Chrysanthou, & Koutidou, 2012). Thus, it is decisive to ensure that the content of volatile compounds remains almost unchanged during freezing to achieve reliable results in aroma research. The present literature concerning the evolution of the volatile compounds during storage has been focused on the changes of the aroma profile at room temperature (Chiavaro, Vittadini, Musci, Bianchi, & Curti, 2008; Jensen, Oestdal, Skibsted, Larsen, & Thybo, 2011; Jensen, Ostdal, Skibsted, & Thybo, 2011; Latou, Mexis, Badeka, & Kontominas, 2010; Plessas et al., 2008, 2011). The extension of shelf-life is one of the biggest challenges for the baking industry today, since the short shelf-life of bread has caused considerable economic losses annually (Plessas et al., 2011). However, as to our knowledge, there is no literature concerning the evolution of volatile compounds during freezing.

Therefore, the aim of the present study was to investigate the frozen storage time suitable to preserve the volatile profile of wheat bread samples, in order to achieve reliable aroma analyses. For this purpose, bread samples frozen for one, two and four weeks were analysed using a static headspace methodology for the very volatile compounds and a solvent extraction methodology for the rest of common volatile compounds studied in wheat bread, both with GC/MS.

2. Materials and methods

2.1. Materials

Strong wheat flour (11.73% and 11.20% w/w of moisture and protein contents, respectively) from Harinera Castellana (Medina del Campo, Valladolid, Spain), ascorbic acid from Sigma Aldrich

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Abbreviations: Anova, analysis of variance; FU, Farinograph Units; GC/MS, gas chromatography–mass spectrometry; RH, relative humidity; SHS, static headspace; SIM, Selected Ion Monitoring.

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(Gillingham, UK), *Saccharomyces cerevisiae* (Saf-instant yeast) from Lesaffre (Lille, France), salt from Ybarra (Sevilla, Spain) as well as tap water were used to make the bread samples. To check the retention time and the mass spectra of the main volatile compounds, the 38 analytical standards listed in Table S1 were purchased from Sigma Aldrich (Gillingham, UK).

2.2. Methods

2.2.1. Bread making and storage conditions

The following ingredients, as % on wheat flour basis, were utilized: salt (1.8%), instant yeast (1%), ascorbic acid (0.01%) and water (52.7%, calculated to obtain 500 Farinograph Units, FU). The dough was made with 1500 g (±0.05 g) of flour and the amount of water was adjusted to an average moisture content of 12%. The ingredients were mixed using a Kitchen-Aid Professional mixer (KPM5, KitchenAid, St. Joseph, Michigan, USA) for 15 min at speed 2. Six pieces of dough of 500 g each were rounded and left for fermentation for 90 min in a chamber at 30 °C with 75% RH. Later, the pieces of fermented dough were baked at 180 °C for 40 min and left for 30 min to reach room temperature. One piece of bread was analysed as freshly prepared sample (day 0), as a baseline for comparison over time. First, the fresh bread was cut into slices of 5 cm long and then the crumb was separated 1 cm from the crust, to avoid contamination of the crumb with crust volatile compounds. Then, the crumb was frozen with liquid nitrogen and ground in an Ika grinder model M20 (Staufen, Germany) for 10 s. Finally, 50 g of the powder was submitted to volatile compounds analyses (Subsection -2.2.2). The crumb of another piece of bread was separated from the crust in the same way, ground and frozen with liquid nitrogen, as was reported by studies that freeze the crumb separated from the crust until their analysis (Bianchi et al., 2008; Paraskevopoulou et al., 2012). The crumb powder was separated into three aluminum packets placed in polyethylene bags and frozen at −21 °C for one, two and four weeks, respectively, until their volatile compounds were analysed. Finally, another piece of bread was taken as a control sample of the evolution of the volatile compounds over time at room temperature, in order to compare the changes during freezing with conventional room storage. It was stored in a laboratory oven, wrapped in aluminum foil, at a controlled temperature of 22 °C. This bread was stored with the crust in order to protect the volatile compounds from dramatic losses that could prevent the analysis of changes due to the natural aging of bread. After one week, the crumb was separated from the crust, frozen with liquid nitrogen and grinded and finally submitted to volatile compounds analyses (as was explained for the fresh bread). Experiments were made with a piece of bread stored for two weeks at room temperature, but the volatile compounds analysis was not accomplished because the bread was completely stale. All the samples were thawed for 30 min before the aroma analyses were conducted. The whole experiment was conducted in duplicate (n = 2).

2.2.2. Volatile compounds analyses: solvent extraction, static headspace & GC/MS

The fresh sample, the control sample (stored one week at room temperature) as well as the three frozen samples (one, two and four weeks) were analysed following the solvent extraction methodology for the analysis of volatile compounds in wheat bread crumb developed by our research group (Pico, Nozal, Gómez, & Bernal, 2016). This is considered a suitable method to examine the possible changes in the volatile compounds of frozen crumb since the reported limits of detection have been lower than 35 μg kg^{-1}. Each sample was analysed in duplicate (n = 2).In order to evaluate the changes of the very volatile compounds, static headspace analyses of ethyl acetate and ethyl alcohol were performed, which eluted with the solvent in the lipases method. Thus, 1 g (±0.050 g) of each sample was placed in a 20 mL vial and sealed with a septum cap. The samples were then extracted for 90 min at 90 °C, without agitation, in a Static Headspace autosampler 7694 from Hewlett Packard (Palo Alto, California, USA). The loop and transfer line temperatures were 100 °C and 105 °C, respectively. The carrier gas employed was helium, supplied by Carburos Metálicos (Barcelona, Spain), with a carrier gas pressure of 23 psi. The vial pressurization was 14 psi for 0.2 min. The loop filling time was 0.2 min, the equilibration loop time was 0.05 min and the injection time was 1 min. Each sample was analysed in duplicate (n = 2). GC–MS conditions for the solvent extraction methodology are the same that those previously described (Pico et al., 2016). Specifying the gradient conditions, for the solvent extraction methodology the temperature ranged from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, after which the temperature was increased to 114 °C (3 min) at 6 °C/min, and then to 136 °C (0 min) at 1.5 °C/min. Finally, the temperature was raised to 245 °C at 85 °C/min. This temperature was held for 25 min in order to elute the hydrolysed fat (glycerol and free fatty acids). For static headspace (SHS) analyses, the temperature ranged from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, and afterwards the temperature was increased to 114 °C (6.7 min) at 1 °C/min. Analyses were performed in Selected Ion Monitoring (SIM) mode and the 38 volatile compounds were identified and confirmed by comparison of their retention times and mass spectra (target and qualifier ions) with standards (Table S1) and with the Mass Spectra Library (Wiley 7N edition).

2.2.3. Data analysis

The One-way Analysis of Variance (Anova) of the peak areas (n = 4, each bread prepared in duplicate and analysed in duplicate) was computed by the software Statgraphics Centurion version XVII (Statpoint Technologies, Warrenton, Virginia) with statistical significance set at $p < 0.05$.

3. Results and discussion

3.1. Evolution of the volatile compounds during storage at room temperature

A total of 38 volatile compounds reported as main volatile compounds in fresh wheat bread (Birch, Petersen, & Hansen, 2014; Chiavaro et al., 2008; Jensen, Oestdal, et al., 2011; Latou et al., 2010; Makhoul et al., 2015; Plessas et al., 2008, 2011) were selected to examine the evolution during room temperature and frozen storages (Table S1). The results of the 38 selected volatile compounds for the fresh sample and the control sample stored one week are summarised in Table 1. Nearly all of the volatile compounds, disregarding the boiling point, polarity or functional group, decreased after one week of storage at room temperature. Only for 2,3-butanedione, 1-pentanol and 1,3-butanediol there were no significant differences between the fresh sample and the stored sample, although they were present in low amount. This general tendency of volatile compounds to decrease after a few days of storage at room temperature has been commonly reported (Chiavaro et al., 2008; Jensen, Ostdal, et al., 2011; Plessas et al., 2011). These changes have been attributed to evaporation, staling of bread or oxidation reactions, although they have not been explained. Chiavaro et al. (2008) found in their study that the volatile compounds in the wheat bread crumb decreased between 1.5 and 3 times after 8 days of storage at 25 °C, which is in concordance with the average decrease of 2.75 times (39% of losses) of our study. Ethyl acetate as well as ethyl alcohol also showed large decreases of 65 and 70%, respec-

Table 1
Peak area of the target ions ($\times 10^6$) of the 38 volatile compounds studied in the crumb of the fresh bread and the bread stored for one week at 22 °C. Standard deviations (SD) are given after ± (n = 4). Different letters in the same row indicate significant differences in One-way Anova (95% significance level).

Volatile compounds	Fresh	1 week	% losses[b]	p-value
Ethanol[a]	39.332 b ± 4.720	11.651 a ± 1.305	70	0.0026
Ethyl acetate[a]	6.345 b ± 0.318	2.243 a ± 0.067	65	0.0012
2,3-Butanedione	3.354 a ± 0.323	2.320 a ± 0.157	31	0.0553
1-Propanol	1.179 b ± 0.065	0.516 a ± 0.021	56	0.0053
2-Methyl-1-propanol	6.715 b ± 0.102	0.882 a ± 0.020	87	0.0002
Hexanal	12.671 b ± 0.129	7.705 a ± 0.032	39	0.0004
3-Penten-2-ol	4.069 b ± 0.103	3.140 a ± 0.028	23	0.0066
2-Methyl-1-butanol	4.942 b ± 0.032	1.841 a ± 0.011	63	0.0001
3-Methyl-1-butanol	9.970 b ± 0.095	3.983 a ± 0.056	60	0.0002
1-Pentanol	1.224 a ± 0.019	1.186 a ± 0.014	3	0.1502
Acetoin	7.808 a ± 0.183	12.309 b ± 0.268	−58	0.0026
R-(+)-Limonene	0.019 b ± 0.002	0.0085 a ± 0.0004	55	0.0114
2-Octanone	0.348 b ± 0.017	0.193 a ± 0.001	44	0.0058
1-Hexanol	2.539 b ± 0.034	1.646 a ± 0.018	35	0.0009
Acetic acid	403.680 b ± 1.859	344.030 a ± 5.336	15	0.0045
Furfural	1.817 b ± 0.025	0.470 a ± 0.004	74	0.0002
Methional	0.0066 b ± 0.0003	0.0046 a ± 0.0002	31	0.0198
1-Octen-3-ol	0.196 b ± 0.002	0.113 a ± 0.006	42	0.0031
Nonanal	0.863 b ± 0.007	0.604 a ± 0.025	30	0.0050
2,3-Butanediol	203.014 b ± 4.621	162.075 a ± 7.192	20	0.0211
2-Ethyl-1-hexanol	0.444 b ± 0.003	0.030 a ± 0.001	93	0.0000
Isobutyric acid	20.648 b ± 0.139	15.903 a ± 0.743	23	0.0125
Benzaldehyde	0.136 b ± 0.004	0.094 a ± 0.002	31	0.0057
1,2-Propanediol	12.571 b ± 0.194	10.569 a ± 0.491	16	0.0331
Ethyl octanoate	0.259 b ± 0.008	0.132 a ± 0.001	49	0.0021
5-Methyl-2-furaldehyde	1.041 b ± 0.011	0.133 a ± 0.003	87	0.0001
Butyric acid	4.651 b ± 0.022	3.596 a ± 0.183	23	0.0149
Butyrolactone	1.177 b ± 0.025	0.825 a ± 0.015	30	0.0034
2-(*E*)-Nonenal	1.751 b ± 0.006	1.395 a ± 0.080	20	0.0244
3-Methylbutanoic acid	5.054 b ± 0.007	3.960 a ± 0.121	22	0.0061
2-Methylbutanoic acid	1.568 b ± 0.002	1.156 a ± 0.044	26	0.0058
Phenylacetaldehyde	0.059 b ± 0.000	0.022 a ± 0.002	62	0.0014
1,3-Butanediol	0.848 a ± 0.020	0.733 a ± 0.072	14	0.1627
Hexanoic acid	18.920 b ± 0.019	14.855 a ± 0.777	21	0.0178
Benzyl alcohol	1.782 b ± 0.041	1.494 a ± 0.039	16	0.0186
Phenylethyl alcohol	13.132 b ± 0.303	11.589 a ± 0.111	12	0.0212
2,4-(*E*,*E*)-Decadienal	0.206 b ± 0.001	0.056 a ± 0.003	73	0.0002
4-Vinylguaiacol	7.876 b ± 0.048	5.965 a ± 0.154	24	0.0036

[a] Ethyl alcohol and ethyl acetate were analysed by SHS-GC/MS. The other 36 volatile compounds were analysed by solvent extraction and GC/MS.

[b] % losses calculated compared to the fresh sample. Negative values imply that the peak area of the target ion increased compared to the fresh crumb.

tively, which can mainly be explained by their low boiling points. This decrease in the concentration of ethyl alcohol has been also reported by Plessas et al. (2008, 2011). However, there are some controversies with ethanol, since Latou et al. (2010) reported an increase after four days in the concentration of ethanol, although no explanation was given for this outcome. Acetoin was the only volatile compound that showed significant differences with an increase in the peak area after one week of storage at room temperature, which is in concordance with the work of Jensen, Oestdal, et al. (2011). Acetoin is mainly formed from the glycolysis of pyruvic acid in fermentation (Martínez-Anaya, 1996) by the yeast (Capozzi et al., 2016) and it can also be generated by Maillard reactions during baking (Poinot et al., 2010). However, Maillard processes typically do not occur during storage as a consequence of the moderate temperatures. Nevertheless, Birch, Petersen, and Hansen (2013) reported that acetoin can be formed from the decarboxylation of 2-acetolactate, which could tentatively proceed at room temperature. 2-ethyl-1-hexanol, 2-methyl-1-propanol and 5-methyl-2-furaldehyde presented losses of around 90% in the peak area of the fresh bread, which was surprising for 2-ethyl-1-hexanol and 5-methyl-2-furaldehyde regarding their high boiling points. 2-methyl-1-butanol and 3-methyl-1-butanol also presented high losses, around 60%. Coincidentally, those were five of the seven volatile compounds that contained a methyl/ethyl radical group that could lead to some form of steric hindrance, making the interaction difficult between the volatile compounds molecules and the bread matrix. The interactions between the volatile compounds and starch have been attributed mainly to the amorphous fraction of starch, the amylose fraction (Arvisenet, Le Bail, Voilley, & Cayot, 2002). This interaction is based on the generation of complexes, the volatile compound being the ligand that induces the formation of amylose single helices, normally of six glucoses per turn (Rappenecker & Zugenmaier, 1981). As a consequence, the methyl/ethyl radical of 2-ethyl-1-hexanol, 2-methyl-1-propanol, 5-methyl-2-furaldehyde, 2-methyl-1-butanol and 3-methyl-1-butanol could have the potential to lead to high steric hindrances that complicated their access to the amylose helix and made the interaction difficult between the hydroxyl groups of the volatile compound and the amylose of starch. Although 3-methylbutanoic acid and 2-methylbutanoic acid also contained the methyl radical, the high polarity of the acidic group could have the ability of retaining them in the crumb matrix via linkages to the starch by hydrogen bonds (Le Bail, Biais, Pozo-Bayón, & Cayot, 2004). In the case of 1-propanol, the low boiling point justified a loss of 56%. However, the losses higher than 50% of R-(+)-limonene, furfural, ethyl octanoate, phenylacetaldehyde and 2,4-(*E*,*E*)-decadienal could be explained by a combination of steric hindrance together with a low polarity that could force the volatile compounds to be easily released from the matrix. The other 25 volatile compounds presented losses lower than 25% that were perfectly explained by the storage time.

3.2. Changes in the volatile profile during frozen storage

3.2.1. Evolution of the volatile compounds over the four weeks of freezing

The results of the 38 selected volatile compounds for the samples frozen for one, two and four weeks are provided in Table 2. Only 1-propanol and 4-vinylguaiacol showed no significant differences, as they remained almost constant during the freezing experiment. Regarding the first week of freezing, overall there was a decrease in concentration of the volatile compounds, with an average loss of 32% for the volatile compounds using the solvent extraction methodology and 21% and 19% for ethyl acetate and ethyl alcohol, respectively. Nevertheless, in comparison to the control sample, the freezing achieved an average preservation of 34% after one week. Furthermore, delving into more detail, in the sample stored at room temperature, 2-methyl-1-propanol, 1-propanol, 3-methyl-1-butanol, ethyl alcohol, 2-methyl-1-butanol, ethyl acetate and hexanal experienced losses that were 79%, 56%, 54%, 51%, 50%, 44% and 17% higher, respectively, than the frozen sample after one week. This finding could be attributed to their high volatility. Furfural and 2,4-(*E*,*E*)-decadienal also showed losses that were 25% and 73% higher in the room temperature sample than in the frozen one, but they do not present low boiling points. 2,3-Butanedione was the only volatile compound that was better preserved at room temperature than during freezing, which was very surprising taking into account that 2,3-butanedione presents the lowest boiling point of the studied volatile compounds. Nonetheless, 2,3-butanedione can be generated from the oxidative decarboxylation of 2-acetolactate (Birch et al., 2013), a reaction that hypothetically is more spontaneous at room temperature. Alternatively, acetoin showed the same behaviour during freezing as at room temperature and increased after one week of storage but in a lesser degree. This can also be theoretically attributed to the possible deceleration of the decarboxylation of 2-acetolactate upon freezing. For the other 27 volatile compounds there were slight differences between storage at room temperature and freezing, with the differences lower than 15% attributable to the fluctuations of the GC/MS instrument in the measurement between different days (interday repeatability). Thus, the crust of the control sample seemed to act as an efficient protector of these 27 volatile compounds that differed minimally from the frozen samples.

As depicted in Fig. 1, the total content of alcohols, acids and ketones followed the same general tendency during freezing, with a decrease in the total area during the first week, an increase during the second week and a final decrease leading up to the fourth week. Regarding the SHS-GC/MS analyses (Table 2), ethyl acetate also showed a decrease during the first week but then a constant increase leading up to the fourth week. For ethanol, there also was a decrease in the first week but a great increase in the second week (much higher than the fresh sample) and a slight decrease leading up to the fourth week. This indicates that the key differences between the evolutions of the volatile compounds during freezing were achieved during the second week. 2-methyl-1-propanol, 2,3-butanediol, ethanol, 3-penten-2-ol, 2-methyl-1-butanol, benzyl alcohol, phenylethyl alcohol, ethyl acetate, acetoin as well as isobutyric acid, butyric acid, 3-methylbutanoic acid and 2-methylbutanoic acid, were compounds that clearly increased from the first to the second week of freezing. Conversely, 3-methyl-1-butanol, 2-octanone, 1-hexanol, 1-octen-3-ol, hexanoic acid and 2,4-(*E*,*E*)-decadienal remained almost constant during the second week of freezing. The other 17 volatile compounds experienced the expected decrease during the second week. These behaviours of increasing, decreasing or staying constant over the storage time instead of only decreasing, have been also reported by Jensen, Oestdal, et al. (2011), although at room temperature and over three weeks. Considering the outcomes during the first and second weeks from Jensen, Oestdal, et al. (2011), the alcohols showed exactly the same behaviour as in our case, with a decrease in the first week and an increase in the second week up to the initial value of the fresh sample. Although they reported that the content of alcohols did not change significantly, we found significant differences. Regarding the acids, they reported an increase during the first week and a decrease during the second week, while our results showed an increase during the second week and then a decrease again leading up to the fourth week. They explained the increase through the oxidation of the aldehydes, a reaction that tentatively could be susceptible to be delayed as a consequence of the freezing in our study. Furthermore, in our study the peak area of aldehydes decreased over four weeks, which could be justified by their intermediate state of oxidation, along with the possibility of their reduction to alcohols or oxidation to acids. For Jensen, Oestdal, et al. (2011) the aldehydes increased in the first two weeks explained by lipid oxidation, which on one hand was encouraged by the room temperature and, on the other hand, by the addition of soy oil in their recipe. Finally, although in our study the total content of the ketones exhibited the same general behaviours, 2,3-butanedione and acetoin showed opposite behaviours and it would be better to study them individually. Jensen, Oestdal, et al. (2011) reported the same pattern, with a decrease in the content of 2,3-butanedione in the first two weeks and an increase in the content of acetoin in the first week, although in their case acetoin decreased in the second week.

3.2.2. Effectiveness of the freezing on the aroma preservation for analytical purposes

When using the solvent extraction methodology, the average reduction in the content of the volatile compounds after one week of storage at room temperature was 39% while the average reduction after one week of freezing was 32%, therefore supporting the efficacy of freezing for enhanced volatile compound retention. However, in the analysis of ethanol and ethyl acetate by SHS-GC/MS, the average loss at room temperature was 68% but the average loss during freezing was 20%. As ethanol and ethyl acetate presented the lowest boiling points, the logical situation would be higher losses for them than for the volatile compounds of the solvent extraction methodology, as occurred with the sample stored at room temperature. This surprising high content of ethanol and ethyl acetate in the frozen samples is possibly related, somehow, with physicochemical changes. During the staling of bread, the amylose fraction retrogrades in the first hours of storage while the amylopectin fraction retrogrades over days, although staling has been reported to be mainly due to amylopectin retrogradation (Ronda, Caballero, Quilez, & Roos, 2011). The phenomenon of retrogradation implies that the amylose chains exuded from the starch grain are reoriented in parallel and interact with their hydroxyl groups through hydrogen bonds, while the amylopectin molecules are also associated through hydrogen bonds with their hydroxyl groups. This could lead to the hypothesis that the interaction of the volatile compounds with the hydroxyl groups of retrograded starch would be lower. Ronda et al. (2011) reported that the melting enthalpy of the amylopectin recrystallised in crumb after thawing was higher in the bread frozen for one week than in the fresh bread, which means that the frozen bread retrograded quicker. This would entail that the volatile compounds of the frozen bread would be released easier after thawing than in the fresh bread, due to the higher rate of retrogradation during freezing. As to our knowledge, the effect of retrogradation on the interaction between the starch and the volatile compounds during freezing has never been reported. In view of these results, we hypothesised that the explained effect of retrogradation during freezing should be negligible when using solvent extraction methodologies, probably due to the weakening of the hydrogen bonds in presence of the

Table 2

Peak area of the target ions ($\times10^6$) of the 38 volatile compounds studied in the crumb of the fresh bread and the bread frozen for one, two and four weeks at -21 °C. Standard deviations (SD) are given after ± (n = 4). Different letters in the same row indicate significant differences in One-way Anova (95% significance level).

Volatile compounds	Fresh	1 week	% losses[b]	2 weeks	% losses[b]	4 weeks	% losses[b]	p-value
Ethyl acetate[a]	6.345 c ± 0.189	5.000 a ± 0.186	21	5.220 ab ± 0.175	18	5.415 b ± 0.143	15	0.0035
Ethanol[a]	39.332 b ± 3.456	32.000 a ± 3.001	19	44.614 c ± 4.065	−13	41.821 bc ± 4.013	−6	0.0025
2,3-Butanedione	3.354 c ± 0.323	1.606 b ± 0.024	52	0.953 a ± 0.010	72	nd[c]	100	0.0001
1-Propanol	1.179 a ± 0.065	1.381 a ± 0.018	−17	1.415 a ± 0.058	−20	1.425 a ± 0.130	−21	0.0960
2-Methyl-1-propanol	6.715 c ± 0.102	6.187 b ± 0.056	8	6.788 c ± 0.010	−1	4.284 a ± 0.034	36	0.0000
Hexanal	12.671 d ± 0.129	9.938 c ± 0.051	22	9.019 b ± 0.188	29	4.526 a ± 0.209	64	0.0000
3-Penten-2-ol	4.069 c ± 0.103	3.526 b ± 0.047	13	3.952 c ± 0.102	3	0.056 a ± 0.001	99	0.0000
2-Methyl-1-butanol	4.942 c ± 0.032	4.507 b ± 0.018	9	4.859 c ± 0.003	2	3.130 a ± 0.183	37	0.0001
3-Methyl-1-butanol	9.970 c ± 0.095	8.998 b ± 0.058	10	8.951 b ± 0.036	10	5.774 a ± 0.009	42	0.0000
1-Pentanol	1.224 d ± 0.019	1.060 c ± 0.015	13	0.586 b ± 0.009	52	0.314 a ± 0.010	74	0.0000
Acetoin	7.808 a ± 0.183	9.435 b ± 0.064	−21	12.868 c ± 0.161	−65	7.226 a ± 0.379	7	0.0001
R-(+)-Limonene	0.019 c ± 0.002	0.008 b ± 0.001	55	0.0017 a ± 0.0001	91	nd[c]	100	0.0001
2-Octanone	0.348 c ± 0.017	0.2363 b ± 0.0003	32	0.251 b ± 0.005	28	0.130 a ± 0.002	63	0.0001
1-Hexanol	2.539 c ± 0.034	1.912 b ± 0.035	25	2.034 b ± 0.083	20	0.597 a ± 0.006	76	0.0000
Acetic acid	403.680 d ± 1.859	301.126 c ± 3.202	25	319.549 b ± 9.703	21	177.242 a ± 2.381	56	0.0000
Furfural	1.817 d ± 0.025	0.926 c ± 0.005	49	0.755 b ± 0.001	58	0.426 a ± 0.007	77	0.0000
Methional	0.0066 d ± 0.0003	0.0042 c ± 0.0002	37	0.0026 b ± 0.0001	61	0.00118 a ± 0.00003	82	0.0001
1-Octen-3-ol	0.196 c ± 0.002	0.127 b ± 0.002	35	0.131 b ± 0.011	33	0.073 a ± 0.006	63	0.0002
Nonanal	0.863 d ± 0.007	0.504 c ± 0.006	42	0.294 b ± 0.007	66	0.112 a ± 0.004	87	0.0000
2,3-Butanediol	203.014 c ± 4.621	150.509 b ± 1.506	26	208.044 c ± 0.271	−2	113.890 a ± 3.010	44	0.0000
2-Ethyl-1-hexanol	0.444 b ± 0.003	0.025 a ± 0.006	94	nd[c]	100	nd[c]	100	0.0000
Isobutyric acid	20.648 d ± 0.139	13.145 b ± 0.316	36	14.232 c ± 0.551	31	7.769 a ± 0.410	62	0.0000
Benzaldehyde	0.136 d ± 0.004	0.119 c ± 0.004	12	0.105 b ± 0.001	23	0.079 a ± 0.004	42	0.0004
1,2-Propanediol	12.571 d ± 0.194	8.196 c ± 0.099	35	6.226 b ± 0.322	50	3.649 a ± 0.025	71	0.0000
Ethyl octanoate	0.259 b ± 0.008	0.131 a ± 0.006	49	nd[c]	100	nd[c]	100	0.0000
5-Methyl-2-furaldehyde	1.041 d ± 0.011	0.3741 c ± 0.004	64	0.3018 b ± 0.0002	71	0.155 a ± 0.003	85	0.0000
Butyric acid	4.651 b ± 0.022	3.271 a ± 0.028	30	5.314 c ± 0.299	−14	3.197 a ± 0.107	31	0.0004
Butyrolactone	1.177 d ± 0.025	0.801 c ± 0.011	32	0.624 b ± 0.008	47	0.348 a ± 0.014	70	0.0000
2-(*E*)-Nonenal	1.751 d ± 0.006	1.111 c ± 0.002	37	0.847 b ± 0.019	52	0.402 a ± 0.008	77	0.0000
3-Methylbutanoic acid	5.054 c ± 0.007	3.664 a ± 0.011	28	5.949 d ± 0.046	−18	3.775 b ± 0.058	25	0.0000
2-Methylbutanoic acid	1.568 b ± 0.002	1.041 a ± 0.008	34	1.586 b ± 0.049	−1	1.041 a ± 0.037	34	0.0001
Phenylacetaldehyde	0.05909 c ± 0.00002	0.021 b ± 0.001	65	0.0113 a ± 0.0002	81	nd[c]	100	0.0000
1,3-Butanediol	0.848 d ± 0.020	0.719 c ± 0.022	15	0.600 b ± 0.033	29	0.211 a ± 0.001	75	0.0000
Hexanoic acid	18.920 b ± 0.019	14.661 a ± 0.173	23	14.717 a ± 0.250	22	14.277 a ± 0.211	25	0.0000
Benzyl alcohol	1.782 b ± 0.041	1.418 a ± 0.027	20	2.404 c ± 0.089	−35	1.438 a ± 0.015	19	0.0001
Phenylethyl alcohol	13.132 b ± 0.303	11.280 a ± 0.101	14	20.252 d ± 0.140	−54	17.401 c ± 0.348	−33	0.0000
2,4-(*E*,*E*)-Decadienal	0.206 a ± 0.001	0.255 b ± 0.009	−24	0.263 b ± 0.010	−28	0.204 a ± 0.001	1	0.0017
4-Vinylguaiacol	7.876 a ± 0.048	6.301 a ± 0.039	20	6.528 a ± 0.040	17	6.395 a ± 0.345	19	0.1598

[a] Ethyl alcohol and ethyl acetate were analysed by SHS-GC/MS. The other 36 volatile compounds were analysed by solvent extraction and GC/MS.

[b] % losses calculated compared to the fresh sample. Negative values imply that the peak area of the target ion increased compared to the fresh crumb.

[c] nd = not detected.

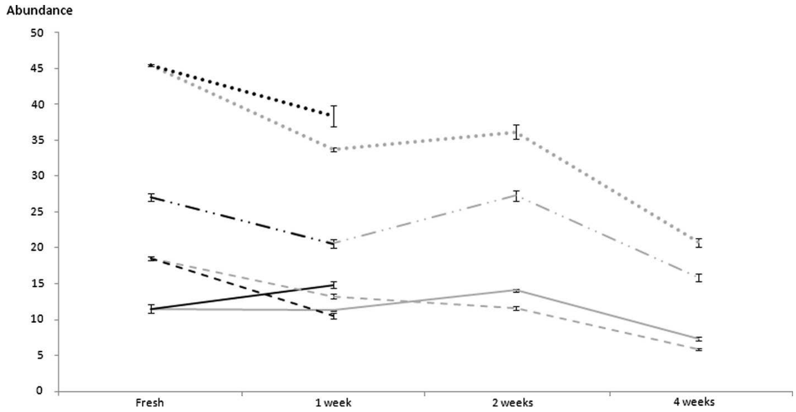

Fig. 1. Evolution of the main groups of volatile compounds in the wheat crumb stored for one week at room temperature (black lines) and in the wheat crumb frozen for one, two and four weeks (grey lines). The results are the sum of the peak areas of the ketones (continuous line, $\times10^6$), aldehydes (discontinuous line, $\times10^6$), alcohols (scratch-doubly spotted line, $\times10^7$) and acids (spotted line, $\times10^7$).

solvent. Thus, the selection of the suitable frozen storage time depends solely on the losses of volatile compounds. In order to avoid losses higher than 24%, the bread samples should be analysed before one week of freezing for solvent extraction analyses. However, when using headspace methodologies the effect should be considered important due to the lack of intermolecular forces with a solvent, leading to an easier release of the volatile compounds from the matrix to the headspace with regard to the fresh bread. As the aim of freezing is to retain the volatile profile of the fresh bread until it is analysed, the easier release of the volatile compounds would disturb the aroma profile, leading to unreliable findings. This explains why the results between the solvent extraction method and SHS-GC/MS were incorrectly closer in the frozen bread than in the fresh one. Therefore, bread samples should be analysed on the same day as preparation if headspace analyses are made. By all means, if freezing is needed, temperatures under −28 °C are suggested in order to decelerate the retrogradation of starch (Ronda & Roos, 2011).

4. Conclusions

The impact of frozen storage time on the volatile profile of wheat bread crumb has been studied for the first time. It has been proven that frozen storage is able to maintain the aroma quality of the bread up to one week (average losses of 24%), with losses of approximately 34% fewer of the volatile compounds regarding room temperature storage (average losses of 58%). The profile of the frozen bread was characterised by losses lower than 20% of alcohols from fermentations such as 2/3-methyl-1-butanol, phenylethyl alcohol or benzyl alcohol and losses higher than 20% of volatile compounds from lipid oxidation like hexanal, 1-hexanol, 1-octen-3-ol, nonanal and 2-(*E*)-nonenal. Moreover, after two weeks of freezing, there was an increase mainly in the content of acids and alcohols. Finally, after one month of freezing, 5 of the 38 volatile compounds were completely lost and the average losses increased up to 53%. Regarding the analytical possibilities in light of these results, bread samples should be analysed before one week of frozen storage time by solvent extraction, in order to avoid losses of volatile compounds higher than 24%. Concerning the static headspace methodology, the samples should be analysed on the same day as the bread is prepared, since the acceleration of the starch retrogradation during freezing could lead to higher releases of volatile compounds in the extraction than those expected in the fresh sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.04.026.

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RESEARCH ARTICLE

Inhibition of fermentation evolution in bread doughs for aroma analyses

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Abstract

The inhibition of the residual fermentation in bread doughs, in order to avoid its evolution, could be crucial to achieve reliable qualitative aroma analyses. Several options have emerged up until now, but they present some drawbacks. In this study, a mixture of methyl octanoate and methyl decanoate (Fames) has been suggested as a non-toxic alternative to the traditional use of mercuric chloride ($HgCl_2$). Rheofermentometric analyses revealed that although $HgCl_2$ is quicker, Fames solution is highly effective in less than 20 min. Moreover, when $HgCl_2$ was added to 90 min fermented dough, it exhibited an unexpected behavior with a high release of CO_2 without the generation of ethyl alcohol, which could affect the dough structure. SHS-GC/MS analyses of ethyl alcohol and 2/3-methyl-1-butanol corroborated the rheofermentometer's results, with a visible reduction in the peak areas and significant differences in the One-way Anova between Fames doughs and blank doughs. The application of the Fames solution to SPME-GC/MS-QTof analyses involved a reduction in the areas regarding the blank without interferences, showing a logical progression of the volatile compounds over the fermentation time, increasing their concentration from 0 to 90 min. This progression was normally lost when the inhibitors were not added, since the yeast acted in an uncontrolled manner due to the changes of temperature during freezing, thawing or chromatographic analyses, leading to wrong aroma results.

KEYWORDS

fatty acids methyl esters, fermentation inhibition, mercuric chloride, rheofermentometer, volatile compounds

1 | INTRODUCTION

It is well known that the aroma of gluten-free bread is not as pleasing as the aroma of bread elaborated with wheat or rye.[1] One of the main ways to improve the weak aroma of gluten-free bread is to understand the evolution of the volatile compounds from the ingredients to the dough during fermentation and to the final baked bread. Thus, it would be possible to understand how the ingredients and bread making conditions are affecting the generation of volatile compounds and then select the most suitable flour, yeast, fermentation time and temperature, etc. The aroma of crumb and crust has been widely studied, but the aroma of the dough has not usually been studied since it is a product in continuous evolution. However, a great number of the volatile compounds decisive in the aroma of the bread crumb are originated through dough fermentation.[2] Therefore, the analysis of the dough aroma should be essential for selecting the best fermentation conditions that improve the final aroma of bread. Nevertheless, the existence of residual sugars after proofing allows the fermentation to carry on if the temperature is suitable. Even if the dough sample has been frozen to stop the fermentation, the low temperature stress is still unclear to make sure how exactly yeast cells die at low temperatures.[3] The cryopreservation of yeast cells depends on the rates of freezing and subsequent thawing, the presence or absence of cryoprotectors such as trehalose or glycerol and the culture growth phase (stationary phase cells are much more freeze resistant).[3] Thus, if the yeast survives the freezing, when the dough is thawed and reaches the room temperature there will be a fermentation evolution that leads to uncertain results in aroma analyses. This means that it

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Abbreviations: DMS, (dimethyl sulfide), Fame C8, (octanoic acid methyl ester), Fame C10, (decanoic acid methyl ester), GC/MS, (gas chromatography/mass spectrometry), GC/MS-QTof, (gas chromatography/mass spectrometry-quadrupole in tandem with time of flight), GC/O, (gas chromatography/olfatometric detector), HPMC, (hydroxy propyl methyl cellulose), SHS, (static headspace), SPME, (solid phase microextraction).

would not be possible to know which volatile compounds were generated during fermentation and which were generated when the sample was going to be analysed. Moreover, this evolution after the fermentation cannot be controlled. However, if the fermentation evolution is inhibited once the established fermentation time is over, then the results would be reliable and the aroma of different fermented samples would be comparable.

Up to now, mercuric chloride ($HgCl_2$) has usually been employed with the aim of successfully inhibiting the fermentation in bread doughs after proofing.[2] Its action is based on the inhibition of glucose metabolism.[4] Nevertheless, in the last few years the use of mercuric compounds has not been recommended, its use will almost certainly be forbidden by the UN Environment Programme's Governing Council in the near future[5] for being highly toxic and cumulative in the environment.[6-9] Perchloric acid (70%) has also been reported as a fermentation inhibitor in bread doughs.[10] However, it is a dangerous, corrosive, unstable acid, as well as a strong oxidant that, in addition to readily forming potential explosive mixtures, could also oxidize the volatile compounds and change the aroma profile.

Several chemical substances have been reported as inhibitors of yeast fermentation in other matrices like wine. Their action is mainly based on: (i) stoppage of cellular replication, with compounds like furfural;[11,12] (ii) inhibition of sugar metabolism, normally with weak acids like formic, acetic, butanoic or propanoic acids;[11,12] (iii) disruption of membrane integrity, with ethyl alcohol[13,14] and with octanoic and decanoic acids or their corresponding esters;[15] (iv) osmotic stress, increasing the sugar substrate concentration.[14] However, furfural, formic acid, acetic acid, propanoic acid, butanoic acid or ethyl alcohol could not be used for fermentation inhibition in aroma analyses since they are compounds commonly analysed in the dough aroma profile.[16] Octanoic and decanoic acids are secondary products of the yeast's metabolism that in the presence of ethyl alcohol and acidic pH are considered fermentation inhibitors, affecting the cell function and diminishing the fermentation of sugars.[15] However, the use of octanoic and decanoic acids could encourage the oxidation of the fatty acids and leads to the increase of volatile compounds from lipid oxidation processes (enzymatic and non-enzymatic). Their esters have been reported not as inhibitory as the free fatty acids, reducing the yeast cell population 23% less than octanoic and decanoic acids.[17] The use of non-toxic alternatives in the inhibition of fermentation evolution could represent also an advantage regarding sensory analyses, since the inhibited dough is a product possible to smell and eat. Its main application can be found for olfatometric analyses (GC/O), since there is a chromatographic separation of the fatty acid esters and the rest of the volatile compounds, avoiding potential interferences in the overall aromatic perception.

Taking into consideration the necessity of analyzing the dough aroma for the improvement of gluten-free bread, the aim of this work has been to evaluate, for the first time, a mixture of methyl octanoate and methyl decanoate (Fames) as an alternative to $HgCl_2$ for inhibiting the residual fermentation after 0 min, 45 min and 90 min of dough proofing. For this purpose, rheofermentometric analyses were performed as well as SHS-GC/MS analyses of main fermentation markers (ethyl alcohol, 3-methyl-1-butanol and 2-methyl-1-butanol). The suggested sample pre-treatment was applied to SPME-GC/MS-QTof aroma analyses of the blank doughs and doughs with Fames added in order to evaluate the impact of the Fames solution in the dough's volatile profile.

2 | MATERIALS AND METHODS

2.1 | Recipe ingredients: Starch, hydrocolloid and yeast

Corn starch (Miwon Daesang, Seoul, Korea), hydroxyl propyl methyl cellulose (HPMC) (Dow Chemicals, Midland, MI, USA) and the dry baker's yeast (*Saccharomyces cerevisiae*) (Lesaffre, Cerences, France) were employed.

2.2 | Standards and solvents

To check the retention time and the mass spectra of the markers of the fermentation inhibition analysed by SHS-GC/MS (section 2.6), the following standards were purchased from Sigma Aldrich (Gillingham, UK): 3-methylbutanol and 2-methylbutanol. Ethyl alcohol was supplied by Panreac (Barcelona, Spain). The chemical compounds employed to stop the evolution of the gluten-free doughs were analytical standards (neat, avoiding esters impurities) of octanoic acid methyl ester (Fame C8) and decanoic acid methyl ester (Fame C10) from Sigma Aldrich (Gillingham, UK) and mercuric chloride ($HgCl_2$) from Panreac (Barcelona, Spain). Finally, in order to check the retention time and the mass spectra of the 35 volatile compounds analyzed in dough by SPME-GC/MS-QTof (section 2.7), all the studied volatile compounds were purchased from Sigma Aldrich (Gillingham, UK). Mixture solutions were prepared in dimethyl sulfide (DMS) (Sigma Aldrich, Gillingham, UK) to achieve the elution of the solvent at the beginning of the chromatogram, avoiding interferences both in the retention time and in the mass spectra.

2.3 | Gluten-free dough-making conditions

The following ingredients as g/100 g on corn starch basis were employed: water (100 g/100 g), sunflower oil (6 g/100 g), sucrose (5 g/100 g), salt (1.8 g/100 g), instant yeast (3 g/100 g) and HPMC (2 g/100 g). They were mixed using a Kitchen-Aid Professional mixer (KPM5, KitchenAid, St. Joseph, MI, USA) for 8 min at speed 2. Six aliquots of 100 g of dough were transferred to aluminum tins: three of them were used as blank and the other three were used to stop the fermentation evolution with a mixture solution of Fame C8 and Fame C10. Another three aliquots of 100 g of dough were transferred to plastic containers to stop the fermentation evolution with a solution of $HgCl_2$, since Hg^{2+} is able to oxidize the aluminum (Al) of the tin to Al^{3+}. The nine aliquots were from the same batch. Then, the dough preparation was made by quadruplicate, two batches for the rheofermentometer analyses and two batches for the volatile compounds analyses by SHS-GC/MS (fermentation inhibition study) and SPME-GC/MS-QTof (aroma analyses).

2.4 | Stop of fermentation evolution prior to rheofermentometer, SHS-GC/MS and SPME-GC/MS-QTof analyses

The procedure has been depicted in Figure S1 in order to facilitate the interpretation of the different steps. Three times were selected to be analysed: 0, 45 and 90 min fermentation. Two aliquots of 100 g of the kneaded dough in aluminum tins were taken. One of them was directly frozen at −20°C (0 min blank). To the second one, 5 mL of the Fames solution (20 g L^{-1} in DMS) were added, softly mixed with a glass rod for 30 sec and frozen at −20°C (0 min Fames). Another two aliquots of 100 g of the kneaded dough in aluminum tins were fermented 45 min at 30°C and a further two were fermented 90 min at 30°C. One of the 45 min fermented and one of the 90 min fermented were frozen at −20°C (45 min blank and 90 min blank) and to the other 45 min / 90 min fermented doughs, 5 ml of the Fames solution were added respectively, softly mixed with a glass rod for 30 sec and frozen at −20°C (45 min Fames and 90 min Fames). Likewise, the same process was followed with the aliquots of 100 g of kneaded dough in plastic containers but with 5 ml of $HgCl_2$ (1.4% in water). Thus, 0 min $HgCl_2$, 45 min $HgCl_2$ and 90 min $HgCl_2$ were obtained. This procedure was repeated in order to duplicate the rheofermentometer analyses (two batches) and the volatile compounds of the doughs by SHS-GC/MS and SPME-GC/MS-QTof (two batches).

The frozen samples were thawed and tempered at room temperature for 30 min before rheofermentometer and volatile compounds analyses.

2.5 | Fermentation evolution measurements: Rheofermentometer analyses

100 g of each dough were introduced in the rheofermentometer F3 (Chopin, Villeneuve-la-Garenne, France), for 60 min at 30°C. The emission (in cm^3) of carbon dioxide (CO_2) was selected to check the efficacy of the selected fermentation inhibitors to inhibit the fermentation evolution. Analyses were made by duplicate (*n* = 2).

In order to evaluate the effectiveness of each solution, three parameters were calculated: (i) the effectiveness at the maximum release of CO_2 (Table 1), which could be related to the amount of yeasts that are deactivated by each solution. It is calculated comparing the height of the inhibited curve with the blank curve at the maximum of the inhibited curve; (ii) the effectiveness after 60 min of measurement in the rheofermentometer (Table 1), which could be associated with the real situation of aroma analyses taking into consideration the time of sampling and of the waiting to be measured. It is calculated comparing the height of the inhibited curve with the blank curve at 60 min; (iii) time employed with each solution in reaching at least 75% of effectiveness (Table 1), which could be related to the speed in stopping the fermentation of each solution. It is the time employed to reduce the CO_2 production of the blank 75%, calculated comparing each height point of the inhibited curve with the blank curve until the point where the reduction is at least 75%.

2.6 | Ethyl alcohol and 2/3-Methy-1-butanol analyses as markers of fermentation inhibition: SHS-GC/MS analyses

1 g (± 0.050 g) of each dough sample was introduced in a 20 ml vial and sealed with a septum cap. After that, the sample was extracted for 90 min at 120°C, without agitation, in a Static Headspace autosampler 7694 from Hewlett Packard (Palo Alto, CA, USA). The loop and transfer line temperatures were 130°C and 135°C, respectively. The carrier gas employed was helium, supplied by Carburos Metálicos (Barcelona, Spain), with a carrier gas pressure of 23 psi and the vial pressurization was 14 psi for 0.2 min. The loop filling time was 0.2 min, the equilibration loop time was 0.05 min and the injection time 1 min. Each sample was analyzed in duplicate (*n* = 2).

GC–MS analyses were performed on a 7890A gas chromatograph coupled to a 5975C single quadrupole mass spectrometer detector equipped with a 7683B automatic injector and a Chemstation 5975C software, all from Agilent Technologies (Santa Clara, CA, USA). Separation was achieved on a polar ZB-Wax column (100% polyethylene glycol, 60 m × 0.25 mm ID × 0.25 μm) obtained from Zebron (Phenomenex, New South Wales, Australia). The GC was operated under programmed temperature conditions: from 45°C (1.5 min) to 100°C (0 min) at 7°C/min, afterwards the temperature was increased to 114°C (6.7 min) at 1°C/min. Total run time was 30 min. The carrier gas was also helium, at a flow rate of 1.1 ml/min. The injector temperature was 250°C working in split mode (1:10). The interface, ion source and quadrupole temperatures were 250°C, 230°C and 150°C, respectively. Analyses were performed in SIM mode, operating in positive electron impact mode with ionization energy of 70 eV. The three analytes were identified and confirmed by comparison of their retention times and mass spectra with pure standards and with the Mass Spectra Library (Wiley 7 N edition).

TABLE 1 Effectiveness of each solution inhibiting the fermentation after 0 min, 45 min and 90 min of fermentation at the maximum release of CO_2 and after 60 min. Time employed with each solution in reaching at least 75% of effectiveness for 0 min, 45 min, and 90 min of fermentation (the effectiveness achieved in % is given in brackets)

	% effectiveness at maximum		% effectiveness after 60 min		Time to reach 75% of effectiveness	
	$HgCl_2$	Fames	$HgCl_2$	Fames	$HgCl_2$	Fames
0 min	91.4	52.6	100	88.9	1.5 min (98.3)	19.5 min (75.6)
45 min	92.9	45.0	99.4	88.4	1.5 min (92.6)	16.5 min (76.0)
90 min	11.5	16.3	6.3	100	25.5 min (75.7)	22.5 min (75.1)

2.7 | Aroma analyses of the doughs: SPME-GC/MS-QTof analyses

0.25 g (± 0.0005 g) of each dough was weighed into a 20 ml vial and sealed with a magnetic screw cap provided with PTFE/silicone septa. 0.25 g was the maximum amount of sample that did not saturate the QTof detector. Since the % RSDs of the intermediate precision were lower than 12%, no internal standard was added. The selected fiber was 50/30 μm DVB/CAR/PDMS (Sigma Aldrich, Gillingham, UK), a polymeric multiphase that allows trapping polar and non-polar compounds.[18] The sample was incubated for 5 min at 35°C (without the fiber) and then extracted for 60 min at 35°C,[18] without agitation. At the end of the extraction time, the fiber was inserted into the GC injector port for thermal desorption during 5 min at 250°C, with an injection volume of 1 μl. Finally, the fiber was conditioned for 30 min at 270°C after each analysis. Each sample was analysed in duplicate (*n* = 2).

GC–MS analyses were performed on a 7890A gas chromatograph coupled to a 7200 Quadrupole-Time of flight (QTof) mass spectrometer detector and MassHunter B.07.00 software, all from Agilent Technologies (Santa Clara, CA, USA). The GC was equipped with a CombiPAL RSI 85 autosampler from CTC Analytics AG (Zwingen, Switzerland) to carry out the SPME analyses. The separation was achieved on a polar Innowax column (100% polyethylene glycol, 30 m × 0.25 mm ID × 0.25 μm) obtained from J&W Scientific (Agilent Technologies, Palo Alto, CA, USA). The GC was operated under programmed temperature conditions: from 45°C (1.5 min) to 100°C (0 min) at 7°C/min, then the temperature was increased to 114°C (6.7 min) at 1°C/min, afterwards it was increased to 136°C (0 min) at 2.5°C/min and finally it was increased to 245°C (5 min) at 85°C/min. Total run time was 43 min. The carrier gas was also helium, supplied by Carburos Metálicos (Barcelona, Spain), at a flow rate of 1.1 ml/min. The injector temperature was 250°C, working in split 1:100 for the most abundant volatile compounds (ethyl acetate, ethyl alcohol, 2-methyl-1-propanol, 2/3-methyl-1-butanol, acetoin, acetic acid, phenylethyl alcohol and 4-vinylguaiacol) and in splitless for the rest of the analytes. For the most abundant compounds it was compulsory to dilute the sample working in split mode because otherwise the signal saturated the detector and it was not possible to perform a correct integration of the peak. However, when the sample was injected in split mode, there were losses of some volatile compounds that were in traces; thus, for the less abundant volatile compounds it was necessary to work in splitless mode in order to achieve their detection. The use of different working modes for different compounds was possible because the same volatile compound was followed among the different samples, which were injected in the same mode. However, different compounds injected in different modes were not compared between them, since they have not been quantified. The interface, ion source and quadrupole temperatures were 250°C, 230°C and 150°C, respectively. Analyses were performed in SCAN mode included a mass range of 20–350 m/z, operating in positive electron impact mode with ionization energy of 70 eV. The 35 analytes (26 minority volatile compounds in Table 3 and 9 majority volatile compounds in Figure 3) were identified and confirmed by comparison of their retention times and accurate mass spectra (with four decimal places) with pure standards and with the Mass Spectra Library (NIST MS Search 2.2 & MS Interpreter). The corresponding Retention index (RI) of the 35 volatile compounds in dough were also calculated and compared with those of the corresponding standards and also with those reported in the literature (Table S1).

2.8 | Data analysis

In order to represent and better interpret the results of the nature of inhibitor employed ($HgCl_2$ or Fames solution) regarding the time of fermentation prior to the inhibitor addition (0, 45 and 90 min), a one-way analysis of the variance of the SHS-GC/MS data of ethyl alcohol, 3-methyl-1-butanol and 2-methyl-1-butanol was calculated. The One-way Anova was computed by the software Statgraphics Centurion (version XVII).

3 | RESULTS AND DISCUSSION

3.1 | Effectiveness in the fermentation inhibition regarding the CO_2 released in the rheofermentometric measurements

Saccharomyces cerevisiae is able to transform 95% of fermentable glucides into ethyl alcohol and CO_2.[16] The rate of the fermentation could be evaluated with the generated amount of both compounds. Thus, the volume of CO_2 generated by the doughs inside the rheofermentometer after the addition of the inhibitor compounds is directly associated with the rate of the residual fermentation.

In Figure 1 are shown the graphics corresponding to the volume of CO_2 released in the rheofermentometer by the blank and the doughs with $HgCl_2$ or Fames added after 0 min (Figure 1A), 45 min (Figure 1B) or 90 min (Figure 1C) of fermentation. Regarding the blank, the volume of CO_2 released is always much higher than that released by the doughs with $HgCl_2$ or Fames added, the success of the inhibition of fermentation with the solutions employed being proved. These differences are more remarkable in the case of the 0 min and 45 min fermented doughs than in the case of 90 min fermented, probably due to the depletion of fermentable sugars after 90 min of previous fermentation. It could be also related to the loss of freeze resistance of the yeast, since yeast cells become more sensitive to temperature changes when they are mixed with the flour.[19] Furthermore, this sensitivity is associated with the stability of the yeast cells when latent or in a metabolically active state. Then, after 90 min of fermentation the yeast cells could be more sensitive and easily die during freezing, reducing the differences between the different doughs.

Regarding the Figure 1A, the non-fermented doughs, the blank reaches a plateau after 13.5 min, related to the fermentation process that is taking place inside the rheofermentometer. However, in the case of the inhibitor solutions, the curve reaches a maximum and afterwards decreases, showing the gradual inhibition of the fermentation. As can be seen in Table 1, at the maximum release of CO_2 (after 7.5 min in the rheofermentometer) the highest reduction of fermentation is achieved with $HgCl_2$ while in the case of Fames is 38.9% lower. Thus, $HgCl_2$ deactivates a higher amount of yeasts, producing less volume of CO_2. This could be explained by the mechanism of inhibition, since $HgCl_2$ is an antifungal considered a potential toxic [4] for yeast while Fames cause the death of the yeast by substrate inhibition.[15]

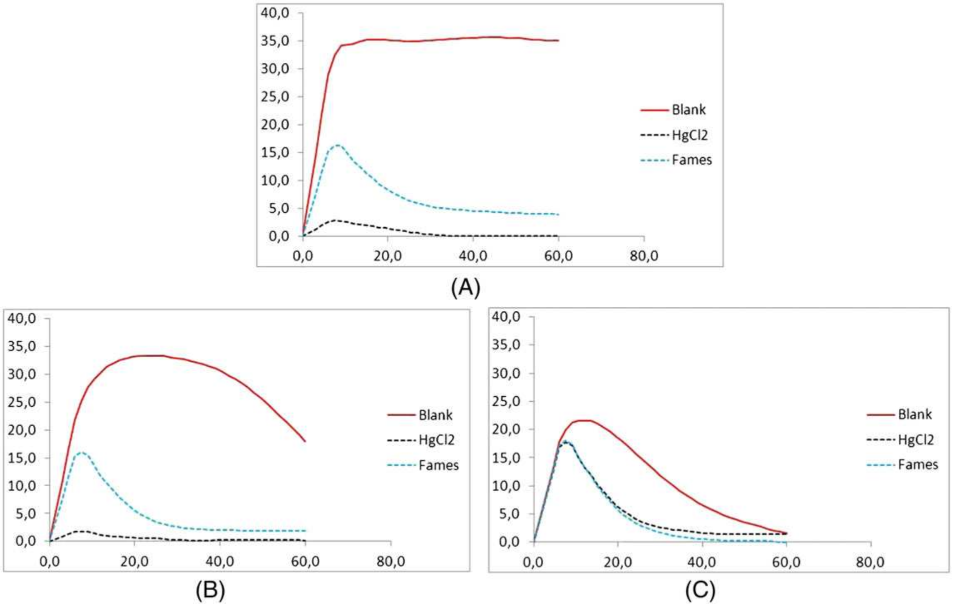


FIGURE 1 Overlapping of the rheofermentometer curves, in cm^3 of CO_2 released (n = 2), of the blank dough (dotted black line), dough with $HgCl_2$ added (continuous grey line), and dough with Fames added (dotted grey line). Non-fermented doughs (A); 45 min fermented doughs (B); 90 min fermented doughs (C)

This means that Fames need more time to inhibit the fermentation, since the yeasts should start to metabolize the fatty acids to be damaged by the overdose of them. However, Fames solution needs only 20 min to achieve 75% of effectiveness (Table 1). Moreover, after 60 min inside the rheofermentometer the effectiveness of Fames is 89% (Table 1). In conclusion for non-fermented doughs, in a short term $HgCl_2$ is more effective than Fames, but $HgCl_2$ is toxic.

Regarding the Figure 1B, the 45 min fermented doughs, $HgCl_2$ and Fames display almost the same CO_2 release curves regarding non-fermented doughs, with a lower final CO_2 volume due to the lesser amount of fermentable sugars after 45 min of fermentation or to the death of the most vulnerable yeast during freezing. This decrease in the final volume is more visible in the blank, which instead of keeping the plateau, reaches the maximum and starts to decrease, although the final volume is much higher than with the inhibitor solutions. Thus, the effectiveness in the maximum and the effectiveness after 60 min (Table 1), are similar to non-fermented doughs but the time to achieve 75% of effectiveness (Table 1), is better now for Fames. Therefore, it could be said that after 45 min of fermentation, the behavior of the yeast is almost the same than in non-fermented doughs when they are inhibited.

Regarding Figure 1C, the 90 min fermented doughs, the release of CO_2 of the blank decreases significantly as was explained before, with a final volume 95% lower regarding the non-fermented dough. In the case of 90 min Fames, the maximum is scantily higher, which means that the effectiveness of the maximum decreases considerably (Table 1), and the time to achieve 75% of effectiveness slightly increases (Table 1). However, the 90 min $HgCl_2$ shows an unexpected behavior: there is a great rise in the maximum release of CO_2, being almost 6 times higher than the non-fermented. Moreover, the final volume after 60 min inside the rheofermentometer increases from almost 0 cm^3 for 0 min and 45 min to 1.5 cm^3 for 90 min (Figure 1), but that of 90 min Fames is 0 cm^3 (Figure 1C). Therefore, the effectiveness after 60 min for 90 min Fames is 100% but in the case of $HgCl_2$ it is almost 5% (Table 1). Moreover, the time to achieve 75% of effectiveness increases by 24 min compared to 0 min $HgCl_2$ and 45 min $HgCl_{2.}$

The upraised release of CO_2 of 90 min $HgCl_2$ would mean high fermentation activities, which would be accompanied by an increase in the concentration of ethyl alcohol and 2/3-methyl-1-butanol. However, as is indicated in Table 2, 90 min $HgCl_2$ exhibits the lowest amount of ethyl alcohol and 2/3-methyl-1-butanol. Therefore, after 90 min of fermentation there may be some changes in the metabolism of the yeasts that lead to high levels of CO_2 without an increase in the concentration of ethyl alcohol and 2/3-methyl-1-butanol. Depending on the conditions of the medium, the yeasts are able to change their metabolism from anaerobic (fermentation) to aerobic (respiration), phenomenon known as Pasteur effect.[20] Once the dough has been fermented for 90 min, the holes of the dough are filled with CO_2 that should be partially released during baking.[21] However, when the dough is frozen, the ice crystals (oxygen carriers) are preferably formed in the gas cells,[22] moving the CO_2. On the other hand, during freezing there is also a creation of ice crystals in the surface of the dough that are melted when the dough is thawed and partially absorbed back into the dough.[23] Finally, the fluctuating temperature of the dough stored implies a great separation of water from starch, water that may not return to its original state resulting in an increase in the water mobility.[24] Thus, a higher amount of freezable water results in a greater number of ice crystals,[25] freezable water that is expected to be increased with the increase of the frozen storage period due to the damage of the starch matrix and the phase-separation of the ice from

TABLE 2 Volatile compounds, markers of fermentation inhibition, found in the blank dough and in the doughs with Fames and $HgCl_2$ added after 0, 45 and 90 min of fermentation (SHS-GC/MS). Results are given in area values of the target ion (x 10^5) (n = 4)

	Ethyl alcohol [a]	3-methyl-1-butanol [b]	2-methyl-1-butanol [c]
Blank 0′	330.37 f	5.47 g	2.90 g
Blank 45'	303.68 e	2.09 e	1.05 d
Blank 90'	353.37 g	3.71 f	2.17 f
Fames 0'	221.14 b	0.78 b	0.46 b
Fames 45'	244.68 c	1.10 d	0.63 c
Fames 90'	330.06 f	2.17 e	1.34 e
$HgCl_2$ 0'	75.46 a	0.32 a	0.11 a
$HgCl_2$ 45'	238.53 c	0.91 bc	0.50 b
$HgCl_2$ 90'	269.76 d	1.02 cd	0.54 b

[a] p–values in the Two-way Anova: Inhibitor (F1), 0.0000; Fermentation time (F2), 0.0000; F1xF2, 0.0000

[b] p–values in the Two-way Anova: Inhibitor (F1), 0.0000; Fermentation time (F2), 0.0000; F1xF2, 0.0000

[c] p–values in the Two-way Anova: Inhibitor (F1), 0.0000; Fermentation time (F2), 0.0000; F1xF2, 0.0000

[a-g / bc/cd] Different letters in the same column indicate significant differences in the One-way Anova (significant level 95%).

the starch system.[26] Thus, the incorporation of oxygen by means of ice crystals could force the yeast damaged by the $HgCl_2$ to change the metabolism from fermentation to cellular respiration. This hypothesis justifies the highest increase in the volume of CO_2 but the lowest amount of ethyl alcohol, since cellular respiration produces CO_2 and H_2O from glucose but it does not produce ethyl alcohol.[27]

3.2 | Effectiveness in the fermentation inhibition regarding the concentration of ethyl alcohol and 2/3-methyl-1-butanol

Ethyl alcohol, 3-methyl-1-butanol and 2-methyl-1-butanol have been considered main volatile compounds from yeast[28] and lactic acid bacteria[29] fermentation in bread. Consequently, they were selected as markers of fermentation inhibition. As they are abundant compounds in fermented doughs, they were analyzed by SHS-GC/MS.

In order to better understand the influence of the inhibitor compounds under different times of previous fermentation, a One-way Anova of the area of the target ions was calculated (Table 2). Both the inhibitor solution and the time of the previous fermentation showed an effect, as well as their interaction (p-value <0.0000). For 0 and 45 min of fermentation, the SHS-GC/MS results (Table 2), were in concordance with the rheofermentometer results (Table 1): the lower the fermentation inhibition, the higher the amount of volatile compounds from fermentation, as in the case of the doughs with Fames solution added. The content of ethyl alcohol showed a significant difference between 0 min $HgCl_2$ and 0 min Fames, but there was no significant difference between 45 min $HgCl_2$ and 45 min Fames. This is linked with the suggested hypothesis of the mechanism of inhibition in section 3.1, since after 45 min of fermentation the yeast is metabolically active and the action of Fames could be quicker. In the case of 3-methyl-1-butanol and 2-methyl-1-butanol, the significant differences varied less than for ethyl alcohol but were similar between them, since both are Ehrlich alcohols. Regarding 45 min $HgCl_2$ and 90 min $HgCl_2$, the difference was slightly significant for 3-methyl-1-butanol but for 2-methyl-1-butanol there was no significant difference, which could be associated with the change of the metabolism after 90 min of fermentation to cellular respiration, releasing CO_2 without generation of volatile compounds. Finally, both $HgCl_2$ and Fames were more effective inhibiting the Ehrlich alcohols than ethyl alcohol.

It could be concluded that, although less effective than $HgCl_2$, the Fames solution is suitable for the inhibition of the fermentation in doughs, avoiding the potential toxicity of the non-recommended $HgCl_2$ and the unexpected behavior of 90 min $HgCl_2$ dough. With the use of Fames there is a visible reduction of ethyl alcohol and 2/3-methyl-1-butanol and significant differences in the One-way Anova regarding the blank. Moreover, when the fermentation evolution is inhibited, there is a logical progression of the volatile compounds over the fermentation time, increasing the amount of volatile compounds with the fermentation. This progression is lost when the inhibitors are not added, since the yeast acts in an uncontrolled manner in the different cycles of temperatures during the freezing, thawing and chromatographic analyses.

3.3 | Impact of fermentation inhibition on the SPME-GC/MS-QTof aroma analyses of the corresponding doughs

In Figure 2 there is an overlapping of the chromatogram of the 90 min blank dough (in red) and the chromatogram of the 90 min Fames dough (in black). Taking into consideration that most of the compounds in dough come from fermentation,[30] the success of the fermentation inhibition is evidenced, since the peak areas of the blank dough are higher than those of the Fames dough. Moreover, there is neither creation nor elimination of volatile compounds: peak number 1 corresponds to diethyl disulfide, an impurity of the DMS solvent, and peak number 5 is an unidentified compound that does not correspond to the volatile compounds of interest. On the other hand, the absence of interferences of the Fames solution in DMS is also demonstrated: methyl octanoate (peak label 2) and methyl decanoate (peak label 6) are not coeluting with analytes of interest, inasmuch as the peak number 3 and 4 of the blank chromatogram corresponds to silane derivatives (identified with the NIST library) that come from the column bleeding or the SPME fiber bleeding. The rest of the small peaks that are higher in 90 min Fames are part of the background and do not correspond to volatile compounds of interest.

35 volatile compounds were identified both in the 0, 45 and 90 min blank doughs and the 0, 45 and 90 min Fames doughs. For an easier interpretation of the results, the most abundant compounds (analyzed in split 1:100 mode) were depicted in Figure 3. All of them have been reported as generated during fermentation[28] and clear decreased in the Fames dough. Moreover, in the Fames doughs the concentration of the analytes increased from 0 min to 90 min of previous fermentation, following a logical progression during fermentation (Figure 3B). However, when the Fames solution was not added and the fermentation continued evolving, the yeast acted in an uncontrolled manner in the different cycles of temperatures, as was the case with the

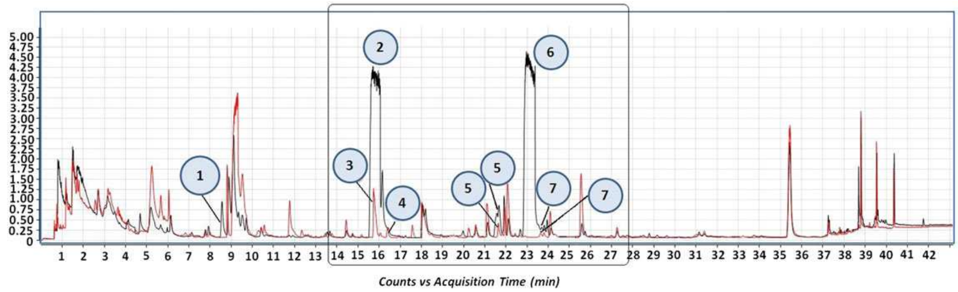
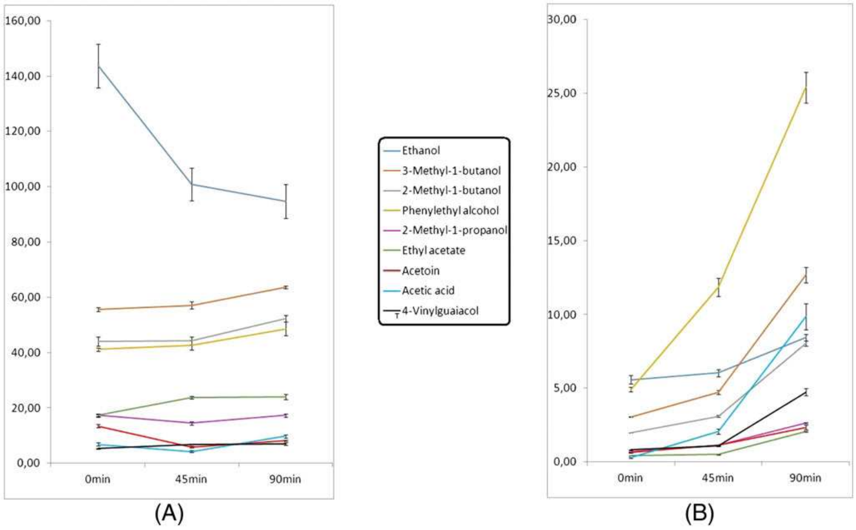


FIGURE 2 Overlapping of the SPME-GC/MS-QTof chromatograms of the blank dough fermented for 90 min (in red) and of the dough with Fames added after 90 min of fermentation (in black). In order to show the lack of interferences of the Fames solution in DMS, only the area of elution of Fame C8 and Fame C10 has been surrounded and explained. Chromatogram in black: (1) diethyl disulfide, (2) methyl octanoate, (5) unknown compound, (6) methyl decanoate, (7) Butyrolactone. Chromatogram in red: (3) Silane derivative, (4) Silane derivative, (5) unknown compound, (7) Butyrolactone



(A)

(B)

FIGURE 3 Evolution of the major volatile compounds at 0, 45 and 90 min of fermentation analysed by SPME-GC/MS-QTof in the blank dough (A) and in the dough with Fames added (B). Results are given in area values (x 10^5), except for ethyl alcohol with Fames added (x 10^6)

SHS-GC/MS analyses of ethyl alcohol and 2/3-methyl-1-butanol. The same occurred in the blank doughs analyzed by SPME-GC/MS-QTof, where only three compounds increased from 0 to 90 min of previous fermentation (Figure 3A). Taking into consideration ethyl alcohol and 2/3-methyl-1-butanol in the blank doughs, they presented different "uncontrolled behavior" between the SHS and the SPME analyses. Those differences cannot be attributed neither to the use of different GC/MS instruments nor the use of different split modes, since the behavior of ethyl alcohol and 2/3-methyl-1-butanol in the doughs with the Fames added was exactly the same, with a continuous increase. The differences could be explained by the different temperature and time that the dough suffered meanwhile it was waiting to be injected or even by the different temperature that the dough experimented in the first minutes of measurement until the yeast died.

The same behavior showed the other 26 minor volatile compounds found in all blank and Fames doughs, analysed in splitless mode and listed in Table 3. Most of the compounds come from fermentation, but there are also some compounds generated by lipid oxidation processes such as heptanal, 1-pentanol, 1-hexanol, 1-octen-3-one and benzyl alcohol.[28] Some of them present Maillard reaction as a possible second source, but it was dismissed due to the low temperatures applied both in dough elaboration and SPME analyses. There was also a general increase in the concentration of the analytes in the Fames doughs from 0 to 90 min of previous fermentation, following again a logical progression during fermentation. Only acetaldehyde and furfural showed a decrease in the signal, probably due to evaporation for acetaldehyde or transformation to furfuryl alcohol for furfural.[31] This justifies why the concentration of furfuryl alcohol keeps constant with the previous fermentation time.

TABLE 3 Minor volatile compounds analysed by SPME-GC/MS-QTof in the blank doughs (0, 45 and 90 min of fermentation) and in the doughs with Fames added (after 0, 45 and 90 min of fermentation). Results are given in area values of the target ion (x 10^5). Standard deviations (SD) are given after ± (n = 4)

	Blank 0 min	±	Blank 45 min	±	Blank 90 min	±	Fames 0 min	±	Fames 45 min	±	Fames 90 min	±
Acetaldehyde	13.646	1.149	22.789	1.960	22.993	1.957	7.559	0.636	6.613	0.569	5.239	0.446
Heptanal	0.993	0.038	14.935	0.092	12.763	0.284	0.304	0.012	1.401	0.009	1.614	0.036
Ethyl hexanoate	22.513	0.020	17.128	1.195	13.851	0.489	0.771	0.001	5.712	0.398	26.654	0.942
1-Pentanol	5.288	0.262	3.580	0.026	3.536	0.101	0.691	0.034	0.859	0.006	1.207	0.034
2-Octanone	2.763	0.073	3.335	0.020	2.604	0.042	1.043	0.028	1.228	0.007	2.045	0.033
6-methyl-5-hepten-2-one	3.386	0.240	3.604	0.130	2.629	0.140	1.157	0.082	1.487	0.054	1.809	0.097
1-Hexanol	40.495	1.616	37.018	0.912	36.606	1.181	11.117	0.444	13.370	0.329	20.368	0.657
Ethyl octanoate	38.346	0.462	35.607	2.076	30.705	1.007	1.311	0.016	11.996	0.699	58.639	1.924
1-Octen-3-ol	4.567	0.062	23.321	0.900	21.153	0.551	2.606	0.035	3.218	0.124	5.782	0.151
4-methyl-1-hexanol	11.076	0.023	11.783	0.087	13.063	0.062	6.720	0.014	11.313	0.084	23.078	0.109
Furfural	7.177	0.137	1.154	0.063	0.906	0.033	3.966	0.076	3.105	0.169	2.410	0.089
2-ethyl-1-hexanol	6.186	0.049	6.506	0.039	5.837	0.020	6.267	0.049	9.795	0.058	12.334	0.042
Benzaldehyde	2.429	0.024	1.871	0.001	1.777	0.009	2.117	0.021	2.135	0.001	2.177	0.011
Propanoic acid	13.309	1.056	74.380	8.033	72.453	6.788	8.486	0.674	24.430	2.639	32.197	3.017
Isobutyric acid	38.005	0.037	29.608	2.289	50.986	1.996	16.066	0.015	37.278	2.882	44.593	1.745
Butyrolactone	3.181	0.077	1.914	0.027	1.439	0.027	0.701	0.017	0.813	0.011	1.364	0.026
Ethyl decanoate	6.148	0.116	4.068	0.226	2.197	0.066	0.208	0.004	1.360	0.076	4.231	0.127
Butyric acid	12.514	0.195	65.395	8.365	66.896	4.799	6.574	0.102	19.946	2.552	31.558	2.264
Phenylacetaldehyde	4.364	0.108	9.346	0.340	9.225	0.282	0.631	0.016	2.321	0.084	5.420	0.166
Ethyl benzoate	4.339	0.199	3.211	0.066	2.495	0.083	5.140	0.236	5.716	0.118	5.875	0.195
Furfuryl alcohol	1.785	0.025	0.915	0.072	0.897	0.042	1.713	0.024	1.736	0.137	1.706	0.079
2-Methylbutanoic acid	16.705	0.546	84.006	8.867	84.561	5.845	6.455	0.211	20.286	2.141	22.281	1.540
3-Methylbutanoic acid	23.449	1.202	81.633	7.739	81.653	5.964	8.976	0.460	23.904	2.266	30.614	2.236
1.3-Butanediol	2.803	0.021	31.235	3.203	31.874	1.753	0.931	0.007	2.541	0.261	3.217	0.177
Hexanoic acid	49.784	0.560	28.604	3.296	26.304	1.664	45.434	0.511	56.486	6.509	69.010	4.364
Benzyl alcohol	1.304	0.008	1.365	0.002	1.169	0.004	1.037	0.006	1.250	0.002	1.433	0.005

Therefore, it is concluded that the addition of the Fames solution to the doughs for inhibiting the evolution of the fermentation leads to reliable and logical aroma analyses, controlling the yeast action and allowing the study of the evolution of volatile compounds during fermentation.

4 | CONCLUSIONS

Rheofermentometric analyses of the doughs revealed that for inhibited doughs with 0 min or 45 min of previous fermentation, $HgCl_2$ was quicker but after 20 min Fames could achieve effectiveness higher than 75%. Nonetheless, the One-way Anova of the SHS-GC/MS analyses showed significant differences between Fames and the corresponding blank, always with smaller peak areas in the Fames doughs. For 90 min of previous fermentation, $HgCl_2$ presented an unexpected behavior with a great increase of the CO_2 release without generation of ethyl alcohol, which could affect the dough structure. Finally, when the Fames solution was added, the SPME-GC/MS-QTof aroma analyses exhibited a logical progression of the volatile compounds over the fermentation time, increasing their amount from 0 to 90 min without interferences. Thus, Fames solution represents an effective, non-toxic alternative to $HgCl_2$ for inhibiting the fermentation evolution and achieving reliable qualitative aroma analyses of doughs.

Future studies could be focused in the search of non-volatile chemical compounds that do not appear in the chromatogram, avoiding possible interferences in breads elaborated with other flour basis that present other volatile compounds. Moreover, non-volatile compounds would be useful for sensory analyses of the edible dough in order to avoid possible interferences of the Fames mixture, which is volatile, in the overall aromatic perception.

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SUPPORTING INFORMATION

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VII. Section 3

Generation of volatile compounds in gluten-free bread doughs and crumbs. Selection of the most suitable flour/ starch for the improvement of the gluten-free bread crumb aroma

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Evolution of volatile compounds in gluten-free bread: From dough to crumb

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ABSTRACT

Understanding the evolution of volatile compounds from dough to crumb is necessary in order to improve the weak aroma of gluten-free breads. Additionally, sensitive analytical methods are required to detect small changes. In the present study, a solvent extraction method combined with GC/MS was selected to examine the evolution of 31 principal volatile compounds from the beginning of fermentation to the end of baking in maize starch bread. During fermentation, only hexanal, hexanoic acid, benzaldehyde, benzyl alcohol, furfural and furfuryl alcohol remained constant whereas the rest became more abundant. After baking, 2,3-butanedione, 1-propanol, 2-methyl-1-propanol, 3/2-methyl-1-butanol and ethyl octanoate were evaporated whereas the other volatile compounds increased. The alcohols from fermentation, 2,3-butanedione, acetoin, acetic acid, isobutyric acid and ethyl octanoate, were the main volatile compounds in dough; all of them were formed during fermentation. In crumb, alongside those compounds, hexanal, 1-octen-3-ol and nonanal, produced from lipid oxidation, were also important contributors.

1. Introduction

The aroma of bread is one of the main characteristics that influences the customers' choice. There can be no doubt that the ingredients of the recipe should affect the final aroma of bread. In fact, when the bread is elaborated with gluten-free flours, the resulting aroma is weaker than those elaborated with wheat or rye (Pacyński, Wojtasiak, & Mildner-Szkudlarz, 2015). The processes that lead to the final aroma of bread, such as fermentation, lipid oxidation or Maillard reactions, strongly depend not only on the recipe but also on the fermentation and baking conditions (Cho & Peterson, 2010). Thus, it is really important to determine the evolution of the volatile compounds from the 0 min fermented dough to the fermented dough and finally to the baked bread, in order to understand their generation in the different steps. Therefore, the processing of gluten-free breads could be modified to achieve a stronger, improved aroma. However there are only a few studies regarding the aroma of gluten-free breads. Furthermore, they analyse the crumb and crust together without specifying where the volatile compounds come from (Aguilar, Albanell, Miñarro, Gallardo, & Capellas, 2015; Poinot et al., 2009). To our knowledge, there is no research regarding the aroma of gluten-free doughs and its evolution to the crumb. Only one research study regarding the evolution of volatile compounds was found, but in wheat bread (Makhoul et al., 2015).

The selected analytical technique is also important because trace analyses are necessary to detect small differences between the different steps of bread production and, above all, between the different stages of fermentation. Solvent extraction methodologies possess in general lower limits of detection than methodologies using headspace (Pico, Gómez, Bernal, & Bernal, 2016) and allow the detection of a higher number of compounds (Majcher & Jeleń, 2009). The most employed extractant is dichloromethane (Gassenmeier & Schieberle, 1995; Schieberle & Grosch, 1994; Zehentbauer & Grosch, 1998) and only for a few compounds, normally acidic compounds, diethyl ether is also reported (Zehentbauer & Grosch, 1998). Moreover, solvent extraction methodologies have been shown to result in extracts rich in high-molecular weight volatile compounds whereas solid-phase microextraction (SPME) to result in extracts rich in low-molecular weight volatile compounds (Mayuoni-kirshinbaum,

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Abbreviations: ANOVA, analysis of variance; FD, flavour dilution factor; GC/MS, gas chromatography/mass spectrometry; HPMC, hydroxypropyl methylcellulose; OAV, odour activity value; OT, odour threshold; PCA, principal component analysis; PC1, first principal component; SIM, selected ion monitoring.

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Tietel, Porat, & Ulrich, 2012). Thus, SPME may be beneficial for volatile compounds that are very volatile and co-elute with the solvent. On the other hand, solvent extraction treatments are more tedious and need the use of organic solvents (Pico et al., 2016). Therefore, solvent extraction methodologies and headspace methodologies have been frequently employed in a complementary way (Corral, Salvador, & Flores, 2015; Klensporf & Jeleń, 2008; Majcher & Jeleń, 2009). With the purpose of detecting small changes in the concentration of the most important compounds, a solvent extraction method for volatile compounds in bread with low limits of detection was selected.

Therefore, the goal of this study was to understand and explain the evolution of the most common volatile compounds in gluten-free breads from fermented dough after 0 min, 45 min and 90 min to the baked crumb employing a sensitive solvent extraction – GC/MS methodology. In this way it would be possible to tentatively establish conclusions about ways to improve the aroma of gluten-free breads.

2. Materials and methods

2.1. Materials

Maize starch (Miwon Daesang, Seul, Korea), hydroxypropyl methylcellulose (HPMC) (Methocel™ K4M, Dow Chemicals, Midland, MI) and *Saccharomyces cerevisiae* (Saf-instant yeast) (Lesaffre, Lille, France) were used. Sucrose, salt and sunflower oil were purchased from the local market and tap water was employed.

To check the retention time and the mass spectra of the main volatile compounds, the 31 analytical standards listed in Table 1 were purchased from Sigma-Aldrich (Gillingham, UK). The purity of all the standards was higher than 98%.

2.2. Methods

2.2.1. Gluten-free bread making

The following ingredients, as a % on starch basis, were employed: sunflower oil (3%), sucrose (5%), salt (1.8%), instant yeast (3%), HPMC (2%) and water (100%). The dough was elaborated with a basis of 700 g (±0.05 g) of starch and the amount of starch and water was adjusted to an average moisture content of 12%. The ingredients were mixed using a Kitchen-Aid Professional mixer (KPM5; KitchenAid, St. Joseph, MI) for 8 min at speed 56 rpm.

Four aluminum tins were filled with 100 g (±0.05 g) of kneaded dough. 5 mL of a mixture of methyl octanoate and methyl decanoate (20 g L^{-1}, dimethyl sulfide) were added to one of them (0 min fermented dough) in order to inhibit the fermentation evolution (data not shown) and finally it was frozen at −20 °C. The other two were left to ferment for 45 min and 90 min in a chamber at 30 °C with 90% of humidity and then their fermentation inhibition was performed as explained (45 min fermented dough and 90 min fermented dough). The frozen doughs were left at room temperature 30 min before their aroma analyses. The last sample was left to ferment for 90 min and then baked at 190 °C for 40 min. After baking, the gluten-free bread was left at room temperature for 30 min and cut into loaves of 5 cm long. The crumb was separated from 1 cm to crust, to avoid the crumb contamination with crust volatile compounds. Finally, the crumb was ground and frozen at −20 °C. The frozen crumb was left at room temperature 30 min before its aroma analysis. Each dough and crumb was prepared in duplicate (n = 2).

2.2.2. Volatile compounds analyses: solvent extraction & GC/MS

The 0 min, 45 min and 90 min fermented doughs as well as the crumb were analysed following the lipases extraction method (Pico, Nozal, Gómez, & Bernal, 2016): 50 g of each sample were ground with liquid nitrogen and then submitted to a Soxhlet extraction for 5 h at 40 °C with a mixture of diethyl ether/dichloromethane, which contained 25 μL lipase enzyme (Lipozyme CALB L©) in order to hydrolyse the fat. After that, the extract was concentrated by means of a Vigreux column and injected onto the GC/MS. It is a suitable method to examine the evolution of volatile compounds from dough to crumb, since the reported limits of detection were lower than 35 μg kg^{-1}. Analyses were performed in duplicate ($n = 2$).

GC–MS analyses were performed on a 7890A gas chromatograph coupled to a 5975C single quadrupole mass spectrometer detector equipped with a 7683B automatic injector and Chemstation 5975C software, all from Agilent Technologies (Santa Clara, CA). Separation was achieved on a polar ZB-Wax column (100% polyethylene glycol, 60 m × 0.25 mm ID × 0.25 μm) obtained from Phenomenex (Torrance, CA). The gas chromatograph was operated under programmed temperature conditions, ranging from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, after which the temperature was increased to 114 °C (3 min) at 6 °C/min, and then to 136 °C (0 min) at 1.5 °C/min. Finally, the temperature was raised to 245 °C at 85 °C/min. It was held for 25 min in order to elute the hydrolyzed fat (glycerol and free fatty acids). The carrier gas was also helium, at a flow rate of 1.1 mL/min. The interface, ion source and quadrupole temperatures were 250 °C, 230 °C and 150 °C, respectively. Analyses were performed in SIM mode, operating in positive electron impact mode with ionization energy of 70 eV. All the volatile compounds were identified and confirmed by comparison of their retention times and mass spectra (target and qualifier ions) with standards (Table 1) and with the Wiley 7 N edition mass spectral library. Firstly, the standard corresponding to each volatile compound was injected individually in order to unequivocally determine its retention time. After that, the mixture of the 31 standards was injected to check the final retention time. This mixture of standards was injected simultaneously with each sample. The retention time of most of them was slightly different from dough to crumb (1–1.5 min of delay in dough) due to the different matrix and the presence of inhibitor substances in the dough.

2.2.3. Data analysis

In order to better represent and interpret the results of the evolution of each volatile compound in the different samples (0 min, 45 min and 90 min fermented doughs and the crumb), a one-way analysis of variance of the peak areas was calculated using Statgraphics Centurion version XVII (Statpoint Technologies, Warrenton, VA). The total number of replicates was four ($n = 4$), with the dough and crumb prepared in duplicate and, in turn, analysed in duplicate. Principal component analysis (PCA) of the three doughs was calculated with the software Latentix (version 2.00, Latent5), with all GC/MS data autoscaled prior to the analysis.

3. Results and discussion

3.1. Evolution of the main volatile compounds during fermentation

A total of 31 volatile compounds was found and examined to understand their generation and evolution from dough to crumb in maize starch bread, integrating the peak area of each volatile compound. These 31 volatile compounds have been commonly reported in wheat bread dough (Martínez-Anaya, 1996) and crumb (Birch, Petersen, & Hansen, 2014; Pico, Bernal, & Gómez, 2015). Volatile compounds, like 3/2-methyl-1-butanol, benzyl alcohol, phenylethyl alcohol, phenylacetaldehyde, 2,3-butanedione, acetoin and 3-methylbutanoic acid, have been reported to have a positive impact in the final aroma of wheat bread, whereas 1-octen-3-ol,

Table 1
Volatile compounds studied in the 0 min, 45 min and 90 min fermented doughs as well as in the final crumb, in order of elution (Rt, retention time). Target (T) and qualifier (Q1, Q2, +Q) ions employed for each compound are given in the table. The origin of the volatile compounds is also indicated.

Volatile compounds	Rt Standard (min)	Rt Dough (min)	Rt Crumb (min)	T	Q1	Q2	+Q	Origin[a]
2,3-Butanedione	8.570	8.476	7.556	43	31	86	15	F & M
1-Propanol	9.094	8.760	8.216	31	42	59	60	F
2-Methyl-1-propanol	10.036	10.135	9.400	43	41	74	55	F
Hexanal	11.405	12.407	10.906	56	44	72	82	L
3-Methyl-1-butanol	12.479	13.296	12.174	55	70	41	57	F
2-Methyl-1-butanol	12.488	13.304	12.183	57	41	70	29	F
Acetoin	13.911	14.327	13.539	45	88	27	15	F & M
Ethyl lactate	15.472	16.280	15.179	45	75	29	19	F
2-Pentylfuran	17.042	nd	16.746	81	53	82	138	F, L & M
1-Hexanol	18.641	18.168	16.890	56	41	42	55	L
Acetic acid	18.802	17.758	17.011	45	60	15	29	F & M
Furfural	20.772	21.493	20.922	96	39	29	67	F & M
Methional	22.262	23.299	22.827	48	104	76	61	F & M
2,3-Butanediol	22.789	22.187	21.371	45	57	29	75	F & M
1-Octen-3-ol	23.801	24.510	24.068	57	72	43	85	L
Nonanal	22.120	23.453	22.655	57	41	70	98	L
Isobutyric acid	23.002	23.414	22.642	43	41	73	27	F
Benzaldehyde	25.712	26.434	25.864	106	105	77	51	F, L & M
Ethyl octanoate	24.890	25.382	24.280	88	101	127	57	F
Butyric acid	25.449	25.678	25.276	60	73	42	27	F
Butyrolactone	25.676	26.538	25.540	42	28	86	56	F&M
Furfuryl alcohol	25.809	25.809	25.401	98	81	69	53	M
3-Methylbutanoic acid	26.294	26.542	26.201	60	43	87	39	F
2-Methylbutanoic acid	26.298	26.556	26.208	57	74	87	41	F
(*E*)-2-Nonenal	26.484	27.442	27.010	70	55	41	83	L
Phenylacetaldehyde	26.662	27.730	26.544	91	120	92	65	F & M
Hexanoic acid	28.478	30.646	29.832	60	73	87	41	F & L
Benzyl alcohol	28.718	29.648	29.290	79	108	91	51	M & L
Phenylethyl alcohol	29.228	29.930	29.139	91	122	65	77	F & M
(*E*,*E*)-2,4-Decadienal	29.620	29.798	29.377	81	67	95	152	L
4-Vinylguaiacol	31.156	31.715	31.053	150	135	107	77	M

[a] Pico, Bernal, & Gómez (2015). F = fermentation, L = lipids oxidation, M = Maillard.

(*E*,*E*)-2,4-decadienal, benzaldehyde, hexanal and methional have been associated with a negative impact. (*E*)-2-nonenal, 3-methyl-1-butanol, hexanal, 2,3-butanedione and phenylacetaldehyde have been reported as having the highest odour activity values (OAVs). Meanwhile, nonanal and 1-octen-3-ol are important in wheat bread crumb with OAVs higher than 4, and 2-phenylethanol, ethyl octanoate, (*E*,*E*)-2,4-decadienal, acetic acid, methional and 4-vynilguaiacol with flavour dilution (FD) factors higher than 64. Therefore, we hypothesized that these compounds could also appear in the maize starch breads. Among the selected volatile compounds, some come from fermentation (including Ehrlich pathway and glycolysis), lipid oxidation, Maillard reaction (including Strecker compounds) or other origins, as shown in Table 1.

During kneading there is an increase in the enzymatic reactions that result in substrates useful for the generation of volatile compounds (Guinet & Godon, 1994). Maize starch is formed of 70–80% amylopectin and 20–30% amylose, which can be broken down by the combined action of amylases (Robyt, 2009, chap. 7), leading, finally, among others, to maltose units. Yeast species common in breadmaking have saturated kinetics for maltose and also possess α-glucosidases that hydrolyse the maltose into glucose units. Glucose is fast consumed by the yeast while maltose accumulates at first and only starts to be used when the levels of monosaccharides are low (Martínez-Anaya, 1996). The sugar added to the dough is also responsible for the fermentable substrate, which could be partially metabolised by the yeast into glucose and fructose (Martínez-Anaya, 1996), leading to small amounts of volatile compounds during kneading. The amount of proteases and lipases in starch and the rest of the ingredients could be considered negligible; thus the generation of amino acids and free fatty acids due to hydrolysis during kneading could be neglected. Therefore, mainly small amounts of volatile compounds from fermentation were expected in the 0 min fermented dough. On the other hand, the addition of oil to the recipe implied the possible presence of small amounts of volatile compounds from lipid oxidation (Guillén, Cabo, Ibargoitia, & Ruiz, 2005; Poinot et al., 2009), as well as the incorporation of lipoxygenases (Márczy, Németh, Samu, Háger-Veress, & Szajáni, 2002). These lipoxygenases could participate in lipid oxidation processes, generating hydroperoxides during fermentation that are broken during baking, increasing the number of volatile compounds. Moreover, the mixing conditions influenced oxygen incorporation from the air, increasing non-enzymatic lipid oxidation (Gardner, 1975) as well as contact between lipoxygenase and the substrate, resulting in greater amounts of hydroperoxides and oxidation of carotenoid pigments (Martínez-Anaya, 1996) of the sunflower oil.

All of this is in concordance with the volatile profile analysed in the 0 min fermented dough (Table 2), which was characterised by volatile compounds from fermentation, such as 2,3-butanedione, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, acetic acid, 2,3-butanediol, isobutyric acid and ethyl octanoate. These volatile compounds should have been generated in a mild fermentation occurring during kneading and in the brief time needed to inhibit the fermentation with the mixture solution of methyl octanoate and methyl decanoate. All of them have been reported as main volatile compounds in wheat bread dough (Martínez-Anaya, 1996). Small amounts of hexanal, 1-hexanol, 1-octen-3-ol, nonanal and (*E*,*E*)-2,4-decadienal were also found, although only hexanal is expected in wheat dough (Martínez-Anaya, 1996).

When the fermentation time increased to 45 min, apart from the fermentation volatile compounds previously mentioned in the 0 min fermented dough, 1-propanol, acetoin and phenylethyl alcohol appeared to be main volatile compounds, also reported as important aroma compounds in wheat dough (Martínez-Anaya, 1996). These eleven main volatile compounds from fermentation

Table 2
Mean peak areas of target ions (× 10^5) with standard deviations (SD) of 31 volatile compounds studied in 0 min, 45 min and 90 min fermented doughs as well as in the final crumb (n = 4). Different letters in the same row indicate significant differences by one-way ANOVA (significance level 95%). The odour thresholds (OT) of each volatile in water as well as their organoleptic characteristics are also included.

Volatile compounds	0 min dough		45 min dough		90 min dough		Crumb		p-Value	OT[b,c] (μg kg^{-1})	Organoleptic characteristics[d,e,f,g]
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
2,3-Butanedione	17.371 b	0.900	16.938 b	1.044	25.010 c	1.679	10.648 a	0.275	0.0010	6.5	Buttery
1-Propanol	0.885 a	0.007	8.173 b	0.868	21.676 c	1.768	8.351 b	0.159	0.0001	6600	Alcohol-like
2-Methyl-1-propanol	15.050 a	0.827	42.911 b	4.791	78.297 c	7.897	25.535 a	0.089	0.0006	3200	Wine, malty
Hexanal	0.460 a	0.043	0.555 a	0.032	0.674 a	0.048	6.969 b	0.138	0.0000	4.5	Green grass
3-Methyl-1-butanol	13.099 a	0.836	21.995 b	1.399	47.323 d	2.305	33.249 c	0.961	0.0001	250	Balsamic, alcohol
2-Methyl-1-butanol	11.980 a	0.673	17.751 b	0.790	35.005 c	1.409	16.508 b	0.502	0.0001	7000	Fusel, alcoholic
Acetoin	3.460 a	0.229	9.277 b	0.793	23.946 c	1.651	75.660 d	0.779	0.0000	800	Buttery
Ethyl lactate	0.762 a	0.049	0.704 b	0.082	0.903 bc	0.089	1.085 c	0.078	0.0249	14000	Fruity, butter
2-Pentylfuran	0	[a]nd	0	[a]nd	0	[a]nd	8.120	0.423	–	6	Floral, fruity
1-Hexanol	0.167 a	0.009	0.169 a	0.007	0.217 b	0.011	0.511 c	0.007	0.0000	2500	Sweet alcohol
Acetic acid	116.993 a	7.364	206.283 b	11.317	381.075 c	30.531	516.319 d	0.174	0.0001	32300	Vinegar-like
Furfural	0.292 b	0.012	0.275 b	0.029	0.304 b	0.007	0.214 a	0.012	0.0204	3000	Woody, caramellic
Methional	0.032 a	0.003	0.076 b	0.008	0.093 b	0.005	0.534 c	0.022	0.0000	0.04	Boiled potato
1-Octen-3-ol	1.119 a	0.087	2.673 b	0.167	2.254 b	0.152	11.815 c	0.621	0.0000	1	Fruity, buttery
2,3-Butanediol	17.399 a	1.638	89.216 b	10.422	183.904 c	22.200	340.985 d	3.254	0.0001	[h]nf	Mushroom
Nonanal	0.785 a	0.085	2.705 b	0.290	5.942 c	0.714	12.977 d	0.036	0.0000	1	Waxy, green, fatty
Isobutyric acid	34.391 a	2.796	37.886 a	3.696	55.108 b	5.886	82.911 c	0.120	0.0007	8100	Butter, fat, cheesy
Benzaldehyde	0.047 a	0.003	0.057 a	0.002	0.052 a	0.002	0.467 b	0.015	0.0000	350	Bitter almond
Ethyl octanoate	82.868 c	10.129	72.063 bc	6.281	60.400 b	8.306	2.564 a	0.239	0.0013	92	Fruity, floral
Butyric acid	3.631 a	0.495	4.433 a	0.098	6.169 b	0.598	9.621 c	0.172	0.0004	240	Cheesy
Furfuryl alcohol	0.171 a	0.020	0.172 a	0.013	0.171 a	0.015	0.311 b	0.018	0.0000	5000	Sweet, caramel
Butyrolactone	0.173 a	0.013	0.389 b	0.025	0.413 b	0.054	1.082 c	0.039	0.0001	20000	Burnt, sweet
3-Methylbutanoic acid	4.773 a	0.420	4.823 a	0.392	6.361 b	0.931	10.148 c	0.080	0.0017	120	Cheesy, creamy
2-Methylbutanoic acid	1.763 ab	0.184	1.431 a	0.104	1.903 b	0.138	3.831 c	0.062	0.0002	100	Cheesy, acidic
(E)-2-Nonenal	0 a	[a]nd	0.335 b	0.022	1.098 c	0.069	1.342 d	0.018	0.0000	0.08	Cucumber, tallow
Phenylacetaldehyde	0.547 a	0.047	0.730 b	0.039	0.722 b	0.034	0.726 b	0.076	0.0572	4	Floral, rose
Hexanoic acid	5.499 a	0.081	6.266 a	0.248	5.914 a	0.481	13.036 b	0.477	0.0001	3000	Cheesy, fruity
Benzyl alcohol	0.462 a	0.007	0.661 a	0.047	0.655 a	0.056	1.897 b	0.162	0.0003	10000	Fruity, balsamic
Phenylethyl alcohol	3.332 a	0.451	14.925 b	1.537	24.595 c	2.617	73.127 d	2.957	0.0000	1100	Rose-like
(E,E) = 2,4-decadienal	0.092 a	0.004	0.142 a	0.011	0.993 b	0.030	1.186 c	0.072	0.0000	0.07	Fatty, deep-fried
4-Vinylguaiacol	0.953 a	0.086	0.826 a	0.073	1.351 b	0.102	7.888 c	0.040	0.0000	3	Clove

[a] nd = not detected.
[b] Birch, Petersen, and Hansen (2014).
[c] http://www.leffingwell.com/odorthre.htm.
[d] https://pubchem.ncbi.nlm.nih.gov/compound/https://pubchem.ncbi.nlm.nih.gov/compound/.
[e] http://www.thegoodscentscompany.comhttp://www.thegoodscentscompany.com.
[f] http://www.pherobase.comhttp://www.pherobase.com.
[g] Birch, Petersen, and Hansen (2013).
[h] nf = not found.

also arose in the 90-min fermented dough, as expected, except ethyl octanoate. A PCA of the peak areas of the doughs was calculated in order to clarify, as a general tendency, in which stage of the fermentation the volatile compounds are more intensively generated (Fig. 1). It was observed that PC1 explained almost 80% of the variance, which grouped all the volatile compounds in the positive component of the PC1 and only ethyl octanoate in the negative one. Ethyl octanoate was the only volatile compound that decreased during fermentation, while the rest increased, when comparing the 0 min fermented dough with the 90 min fermented. As 90 min fermented dough (positive PC1 of scores plot, Fig. 1a) contained the highest amounts of all volatile compounds (positive PC1 of loadings plot, Fig. 1b, expanded in b1, b2 and b3), they should have been generated in the last part of the fermentation since 45 min and 0 min fermented dough were located in the negative PC1 of scores plot, where only ethyl octanoate was located. Esters in fermentation have been reported to be produced from fatty acid metabolism in the yeast cell as a way to remove the toxic fatty acids (Nordstrom, 1964). Maize starch does not contain an important amount of fatty acids; thus ethyl octanoate may have been produced from the small portion of octanoic acid at the beginning of the fermentation. Depending on the nutrient composition of the medium, that is to say the concentration of acyl Co-A and ethanol and the activity of enzymes, the esterification equilibrium may be displaced to the hydrolysis reaction (Saerens et al., 2008), giving again the acid and the alcohol, probably due to Le Châtelier's principle.

3.2. Evolution of the main volatile compounds from fermentation to baking

The final profile of the maize starch crumb revealed compounds that have been commonly reported in wheat bread crumb (Birch, Petersen, & Hansen, 2013), with a similar profile to wheat bread crumb when pre-ferments are employed (Gassenmeier & Schieberle, 1995). On the other hand, all the volatile compounds analysed in the present study have been reported previously in gluten-free breads that contain maize starch in the recipe (Aguilar et al., 2015; Pacyński et al., 2015), except butanoic acid, 3-methylbutanoic acid, 2-methylbutanoic acid and 2-methylpropanoic acid.

2-Pentylfuran is the only volatile compound that was exclusively generated during baking, since it was not detected in the dough. 2-Pentylfuran has been reported as the most common aroma-active furan in wheat bread crumb with floral-fruity notes (Birch et al., 2014). Although it can be generated by fermentation, lipid oxidation and Maillard reactions (Table 1), it is mainly generated from (E)-2-nonenal during baking (Birch et al., 2014). The other 50 volatile compounds appeared in all the doughs and crumb, most of them being higher in crumb, due in some cases

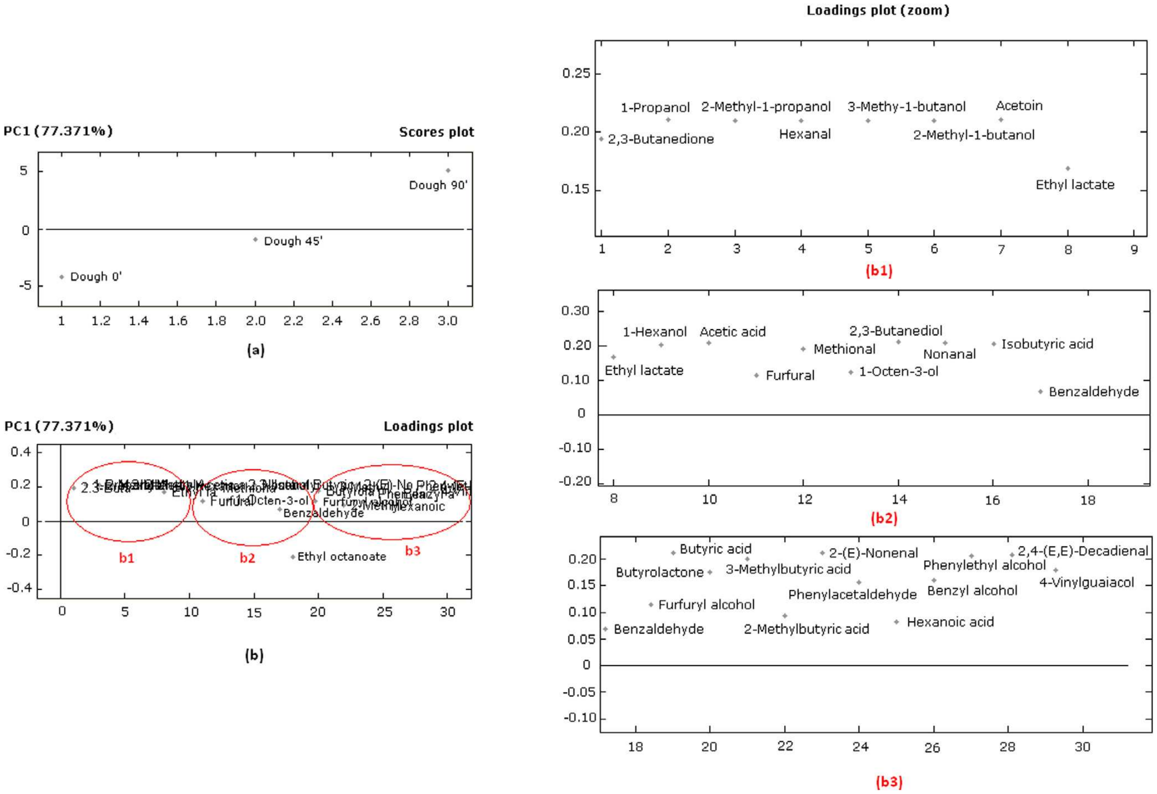

Fig. 1. PCA showing the peak areas (target ion) from volatile compounds of the 0 min, 45 min and 90 min fermented doughs. Scores plot of the dough samples (a), loadings plot of the volatile compounds (b), as well as the expansion of the loadings plot (b1, b2 and b3).

to the development of Maillard reactions, in other cases to the cleavage of the hydroperoxides from the lipid oxidation and also due to the transformation of some compounds catalysed by the heat of the oven.

The results from the one-way ANOVA of the four samples (0 min, 45 min and 90 min fermented doughs and the final crumb) are shown in Table 2. Regarding the significant differences between the doughs and the crumb, 7 different behaviours of the volatile compounds were found as a pattern, as summarised in Table 3.

3.2.1. Volatile compounds that increase during fermentation

Depending on the conditions of the medium, as is reflected in Table 3, there were compounds that increased continuously during fermentation (behaviour B), compounds that increased from 0 min to 45 min of fermentation and then stayed almost constant until the fermentation ended (behaviour D and E) and there were compounds that only increased between 45 min and the end of the fermentation at 90 min (behaviour C).

The alcohols 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol as well as the ester ethyl lactate increased along the entire fermentation process, since they are compounds that are exclusively produced by the yeast activity (Grosch & Schieberle, 1997; Jensen, Oestdal, Skibsted, Larsen, & Thybo, 2011; Martínez-Anaya, 1996; Paraskevopoulou, Chrysanthou, & Koutidou, 2012). Although acetoin, acetic acid,

Table 3
Evolution of the volatile compounds classified according to significant differences in one-way ANOVA. The codes are obtained from the letters assigned in the order 0 min, 45 min and 90 min fermented doughs and the crumb, underlined when both letters are together (from Table 2).

Behaviour from dough to crumb	Code in one-way ANOVA	Volatile compounds
Evaporation from dough to crumb (A)	*bbca; abcb; abca; abdc; cbcba*	2,3-Butanedione, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, ethyl octanoate
Continuous increase from dough to crumb (B)	*abcd, abbcc*	Ethyl lactate, acetoin, acetic acid, 2,3-butanediol, nonanal, (*E*)-2-nonenal, phenylethyl alcohol
Increase from 45 min to 90 min fermentation and during baking (C)	*aabc; ababc*	1-Hexanol, isobutyric acid, butyric acid, 3-methylbutyric acid, 2-methylbutyric acid, 2,4-decadienal, 4-vinylguaiacol
Increase from 0 min to 45 min fermentation and during baking (D)	*abbc*	Methional, 1-octen-3-ol, butyrolactone
Increase from 0 min to 45 min fermentation and then steady (E)	*abbb*	Phenylacetaldehyde
Steady during fermentation, change during baking (F)	*aaab*	Hexanal, benzaldehyde, hexanoic acid, benzyl alcohol, furfuryl alcohol, furfural
Generated during baking (G)	–	2-Pentylfuran

2,3-butanediol and phenylethyl alcohol can be generated by fermentation or by Maillard reactions (Poinot et al., 2010), the yeast activity prevails during fermentation, since the temperature reached in this phase is not enough to develop Maillard reactions. Therefore, these compounds also increased continuously through the fermentation process. Nonanal and (*E*)-2-nonenal, which come from lipid oxidation processes (Birch, Petersen, Arneborg, & Hansen, 2013), also increased from 0 to 90 min of fermentation. This can be attributed to non-enzymatic reactions of lipids oxidation (Gardner, 1975) that could take place during fermentation.

On the other hand, (*E*,*E*)-2,4-decadienal and 1-hexanol are also volatile compounds that come from lipid oxidation processes (Poinot et al., 2010; Quílez, Ruiz, & Romero, 2006), but they were more or less constant in the first part of the fermentation and started to increase from 45 min to 90 min, probably due to non-enzymatic reactions. Likewise, all the acids isobutyric (2-methylpropanoic), 3-methylbutyric and 2-methylbutyric also showed a higher increase in their peak area from 45 to 90 min of fermentation, which could be due to oxidation of the corresponding Ehrlich amino acids (Berry & Watson, 1987), a process that seemed to need more time to take place. Butyric acid showed the same pattern as the other acids, which is produced in the fatty acid synthase pathway in the yeast cell (Berry & Watson, 1987), metabolism that also seemed to need more time to be effective. 2,3-Butanedione, generated through fermentation and Maillard reactions (Poinot et al., 2010), was also more intensively produced between 45 min and the end of the fermentation at 90 min. 2,3-Butanedione can be produced from acetoin *via* glycolysis (Drapon & Richard-Molard, 1979) but can be also enzymatically degraded to 2,3-butanediol (Ehsani, Fernandez, Biosca, Julien, & Dequin, 2009). This explains why it almost stayed constant until 45 min of fermentation, as a consequence of the interaction of both effects. Finally, 4-vinylguaiacol was surprisingly found in dough, since it is mainly generated by Maillard reactions (Pico et al., 2015) during baking. The low amount present in the 0 min and 45 min fermented dough as well as the increase up to 90 min has been reported to be attributed to the activity of feruloyl esterases produced by the yeast (Coghe, Benoot, Delvaux, Vanderhaegen, & Delvaux, 2004), activity that appeared to be more intensive in the second part of the fermentation.

Finally, there were only four volatile compounds that effectively increased at the beginning of the fermentation and stayed constant after 45 min, probably due to the reduction or removal of some precursor, as the amino acid methionine in the case of methional (Frasse, Lambert, Richard-Molard, & Chiron, 1993) or phenylalanine (Jensen et al., 2011) in the case of phenylacetaldehyde. 1-Octen-3-ol (lipid oxidation compound) and butyrolactone (mainly derived from yeast metabolism although it can be also generated by Maillard) also exhibited this behaviour.

3.2.2. Volatile compounds that stay constant during fermentation

The couples hexanal-hexanoic acid, furfuryl alcohol-furfural as well as benzyl alcohol-benzaldehyde did not suffer significant changes in their peak area during fermentation (behaviour F), which could be attributed to a redox equilibrium between them. Thus, furfuryl alcohol has been reported to come from furfural's reduction by the action of *Saccharomyces cerevisiae* (Villa et al., 1992). For its part, hexanoic acid has been reported to derive from the oxidation of hexanal during the lipids oxidation process (Pico et al., 2015) and benzyl alcohol has been disclosed to be generated from benzaldehyde's reduction by the action of alcohol dehydrogenase (Long & Ward, 1989).

3.2.3. Volatile compounds that are evaporated during baking

2,3-Butanedione as well as the alcohols 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol showed a decrease in the peak areas in the crumb as a result of a partial evaporation during baking, a consequence of their low boiling point (behaviour A). While the crumb is below 100 °C, since the water is evaporating with the heat of the oven, the corresponding boiling points are 88 °C for 2,3-butanedione, 97 °C for 1-propanol, 108 °C for 2-methyl-1-propanol and 130 °C for 2-methyl-1-butanol and 3-methyl-1-butanol. Surprisingly, ethyl octanoate and furfural were also evaporated during baking. In the case of ethyl octanoate, since its boiling point is 209 °C, a possible explanation for its loss may be associated with its hydrolysis that could be catalysed by heat (Ramey & Ough, 1980) at the beginning of the baking step. However, furfural is not normally lost during baking. Losses could be attributed to a tiny difference in the solvent extraction efficacy between the dough and the crumb.

3.2.4. Volatile compounds that increase during baking

The other 22 volatile compounds presented a visible increase during baking (behaviour B, C, D and F). Acetoin, acetic acid, 2,3-butanediol, phenylethyl alcohol, benzyl alcohol, furfuryl alcohol, 4-vinylguiaicol, butyrolactone, methional and benzaldehyde can be also generated by Maillard reactions (Jensen et al., 2011; Martínez-Anaya, 1996; Pico et al., 2015; Poinot et al., 2010), which take place with the high temperatures applied during baking and lead to increase in these volatile compounds' concentration. On the other hand, the volatile compounds from lipid oxidation, that is to say nonanal, (*E*)-2-nonenal, hexanal, 1-hexanol, hexanoic acid, benzyl alcohol, benzaldehyde, (*E*,*E*)-2,4-decadienal and 1-octen-3-ol, also increased through baking as a result of the cleavage of hydroperoxides (Guinet & Godon, 1994) generated by lipoxygenases during the fermentation stage.

For their part, all the isoacids (2-methylpropanoic, 3-methylbutyric acid, 2-methylbutyric acid) also showed an increase during baking, which could also be the result of the oxidation of the corresponding Ehrlich amino acids (Birch et al., 2014) at the beginning of the baking, since they are mainly generated through fermentation. Butyric acid also increased during baking, which could be associated to an oxidation of butanal, although butanal was not detected since it should elute during the solvent delay. Similarly, ethyl lactate also increased with the baking step. Although esters are normally generated from fermentation through the fatty acids pathway in the yeast cell (Birch et al., 2014), they can be also generated by the reaction between the acid (lactic acid) and the alcohol (ethyl alcohol) (Frasse et al., 1993). This esterification reaction could take place during baking since the heat could act as a catalyst at the beginning of the baking process.

Finally, phenylacetaldehyde is the only compound that stayed constant during baking, which could be the result of an intensive generation by the Ehrlich pathway (Birch et al., 2013) at the beginning of the fermentation and a poor production through Maillard reactions (Martínez-Anaya, 1996).

3.2.5. Volatile compounds exclusively generated during baking

2-Pentylfuran was the only volatile compound that was not detected in the dough, but only in the crumb. As explained before, it could be generated by fermentation, lipid oxidation and Maillard reactions. However, if it was present in the dough by fermentation, it should have been under the limits of detection of the method. Therefore, we can conclude that it is generated in the crumb due to the high temperatures applied during baking, which lead to Maillard reactions increasing the amount of (*E*)-2-nonenal as a consequence of the cleavage of the hydroperoxides. In fact, its concentration in crumb has been reported to increase with temperature (Birch et al., 2013).

3.3. Implications of this study in the ways of improving the aroma of gluten-free bread

Maize starch dough fermented for 90 min was characterised by the presence of volatile compounds from fermentation, mainly 2,3-butanedione, acetoin, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-propanol, phenylethyl alcohol, acetic acid, isobutyric acid, 3/2-methylbutyric acid and ethyl octanoate. All of them have been reported to have a positive impact on the sensory quality of wheat bread (Pico et al., 2015). Acetic acid improves bread aroma when present in small amounts but it has a negative effect in excessive concentration (Ferreira & dos Reis, 2014, chap. 7). Nevertheless, in moderate concentrations acetic acid could act as suppressor of moulds and ropiness (Ferreira & dos Reis, 2014, chap. 7). 2,3-Butanedione, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-propanol and ethyl octanoate were partially volatilised during baking and only 2,3-butanedione and 3-methyl-1-butanol have been reported with low odour thresholds (OT) (Table 2). Acetoin, acetic acid, phenylethyl alcohol, isobutyric acid and 3/2-methylbutyric increased after baking, but only acetoin and 3/2-methylbutyric have been reported with low-medium OTs. Therefore, most of the volatile compounds from fermentation with pleasant notes were evaporated or have been reported with high OTs (Table 2). In consequence, the fermentation process should be encouraged in order to improve the gluten-free bread aroma. The amount of yeast, the fermentation time and temperature, the kind of microorganism or the reduction of salt are some suggestions that should be studied in gluten-free breads for increasing the fermentation activity.

Volatile compounds from lipid oxidation were also found in maize starch crumb, such as hexanal, 1-hexanol, 1-octen-3-ol, nonanal, (*E*)-2-nonenal and (*E,E*)-2,4-decadienal. Hexanal, 1-octen-3-ol and (*E,E*)-2,4-decadienal have been reported to have a negative impact on the sensory quality of wheat bread (Pico et al., 2015). In the case of nonanal and (*E*)-2-nonenal, there have been some controversies. On the one hand, they have been described as odorants with pleasant aroma properties (Paraskevopoulou et al., 2012) due to their citrus and cucumber notes, respectively. However, on the other hand, they have also been characterised by fatty-tallow notes (Table 2). Only 1-hexanol has been classified with high OTs but the rest have been described as very aroma-active compounds in wheat bread crumb with significantly low OT. In order to decrease the generation of volatile compounds from lipid oxidation and improve the gluten-free bread aroma, the amount of oil is suggested to be reduced as far as possible.

Finally, as it has been shown throughout the manuscript, the volatile compounds found in maize starch dough and crumb have been commonly reported in wheat breads in the literature. Thus, the main differences are likely to be present in the crust and, therefore, the crucial odorants that improve the aroma of gluten-free bread should be found in the crust.

4. Conclusions

Thirty-one volatile compounds, commonly found in the literature of wheat bread, have been detected in the dough and crumb of maize starch-based bread employing a solvent extraction methodology and GC/MS. In the 0 min fermented dough, the most abundant compounds were 2,3-butanedione, 2-methyl-1-propanol, 3/2-methyl-1-butanol, acetic acid, 2,3-butanediol, isobutyric acid and ethyl octanoate. During fermentation there was an increase of 1-propanol, acetoin and phenylethyl alcohol, which after 45 min of fermentation were also abundant. After baking, all of the above-mentioned volatile compounds were notably present in the crumb, but hexanal, 1-octen-3-ol and nonanal were moderately present too. It was also concluded that an increase in the fermentation activity as well as a reduction of the oil content in the recipe may lead to maize starch-based breads with an improved flavour. Since all volatile compounds found in maize starch doughs and crumbs have been previously reported in wheat bread, results indicate that the crucial odorants resulting in gluten-free breads with stronger aroma may come from their crust.

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ORIGINAL ARTICLE

Influence of different flours and starches on gluten-free bread aroma

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Abstract The aim of this research was to study the effect of different gluten-free flours (yellow and white corn, rice, oat, teff, buckwheat, amaranth and quinoa) and starches (wheat, corn and potato) on the generation of volatile compounds in the fermented doughs and crumbs. Volatile compounds were analyzed by static headspace-gas chromatography/mass spectrometry (SHS-GC/MS). Nine fermentation and lipid oxidation volatile compounds were evaluated, which were found to be the same from dough to crumb but vary in levels. Concentrations of compounds produced during fermentation were higher in doughs whereas those from lipid oxidation were higher in crumbs. The type of flour/starch affected the concentration of these volatile compounds. The proportions of ethanol and 2/3-methylbutanol (fermentation compounds) were higher in dough from yellow and white corn, rice and oat while the proportions of hexanal, 1-pentanol and 2,4-decadienal (lipid oxidation compounds) were higher in the doughs made with starches. The proportions of ethanol and 2/3-methylbutanol were higher in quinoa and amaranth crumbs whilst hexanal, 1-pentanol and 2,4-decadienal were higher in yellow and white corn crumbs.

Keywords Volatile compounds · Gluten-free flours · Crumb aroma · Dough aroma · Fermentation · Lipid oxidation

Abbreviations

HPMC	Hydroxymethyl-propyl-cellulose
PCA	Principal component analysis
PC1	First principal component
PC2	Second principal component
SD	Standard deviation
SHS-GC/MS	Static headspace extraction-gas chromatography/mass spectrometry
SIM	Selected ion monitoring
T	Target ion

Introduction

Bread aroma is one of the first characteristics perceived by the human senses, crucial for the acceptance by customers. The most consumed breads have been prepared with wheat or rye flours, which give pleasant notes with compounds that come mainly from fermentation, lipid oxidation or Maillard processes. However, it is well known that if the bread is elaborated with gluten-free flours, its sensory quality decreases in relation to the traditional wheat bread. Celiac people can only consume gluten-free products, which means that they should eat breads with less attractive flavors. Cereal flours such as rice, corn, millet, and teff and gluten-free starches have been commonly employed during gluten-free bread making (Pacyński et al. 2015). Gluten-free breads have been also elaborated with pseudocereals like buckwheat, quinoa or amaranth. Pseudocereals have been reported to contain high nutritional values in terms of proteins, lipids, carbohydrates, vitamins, minerals and fiber

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(Alvarez-Jubete et al. 2010; Hirose et al. 2010; Jancurová et al. 2009). Moreover, they present higher α-glucosidase activity (Elgeti et al. 2014) but lower lipoxygenase activity (Caussette et al. 1997) than conventional wheat flour. Their use has probably not been extended due to their content in saponins, which give bitter taste notes to bread (Oleszek et al. 1999), and also due to their lower availability and higher price.

The differences in proteins, sugars, lipids, enzymes and antioxidants between the different gluten-free flours/starches could lead to important differences in the volatile profile of gluten free breads. Until now, research in gluten-free bread aroma has been focused on the understanding of the origin of the volatile compounds compared to wheat bread (Poinot et al. 2009). There are also a few articles regarding the improvement of gluten-free bread aroma based on the method of baking (Aguilar et al. 2015) or on the addition of sugar-amino acid pairs to encourage Maillard reaction (Pacyński et al. 2015). Nevertheless, as far as we know, there have not been studies of the influence on gluten-free bread aroma of different flours or starches. All the reported articles refer to a mixture of gluten-free flours [rice, corn and buckwheat flours with corn and potato starches (Poinot et al. 2009) or corn starch with chickpea flour (Aguilar et al. 2015)] or to a commercial preparation based on starches (Pacyński et al. 2015).

The aim of this research was to study the effect of different gluten-free flours (yellow and white corn, rice, oat, teff, buckwheat, amaranth and quinoa) and starches (corn, wheat and potato) affect the generation of volatile compounds in the corresponding bread dough and crumb. The volatile compounds generated through the fermentation and lipid oxidation processes were analyzed by static headspace extraction-gas chromatography/mass spectrometry (SHS-GC/MS), since they are considered the main aroma compounds in bread dough and crumb. SHS enables the direct measurement of the ratio of the most abundant compounds in the gaseous phase (Maeda et al. 2009). Both fermented dough and crumb were analyzed in order to understand the influence of bread processing through the subsequent changes of the volatile profile from dough to the related crumb. This could lead to a better understanding of the impact of changing the flour/starch on the aroma of gluten-free breads.

As our knowledge, it is the first time that the aroma profiles of different gluten-free doughs and crumbs elaborated only with one flour or starch have been compared. Knowing the influence of the flour or starch could be essential to producing gluten-free breads with an improved aroma.

Materials and methods

Recipe ingredients: flours, starches, hydrocolloid and yeast

Wheat and potato starches were supplied by Roquette Laisa (Valencia, Spain) and corn starch by Miwon Daesang (Seul, Korea). Wheat flour was purchased from Harinera Castellana (Medina del Campo, España), yellow and white corn flour from Dacsa (Valencia, Spain), rice flour from Molendum (Zamora, Spain), oat flour from Emilio Esteban (Valladolid, Spain) and teff flour from Salutef (Palencia, Spain). Buckwheat, amaranth and quinoa flours were obtained from El Granero Integral (Madrid, Spain). Hydroxyl propyl methyl cellulose (HPMC) was supplied by Dow Chemicals (Michigan, USA) and the dry baker's yeast (*Saccharomyces cerevisiae*) by Lesaffre (Cerences, France). All yeasts belonged to the same batch to decrease the risk of different cell count of yeast and different contaminant bacteria.

Bread making

The following ingredients (as % on flour or starch basis) were used in all the formulas: sunflower oil (6%), sucrose (5%), salt (1.8%), instant yeast (3%), HPMC (2%) and water (100%). The doughs were elaborated with a basis of 700 g (±0.05 g) of each flour or starch and the contents of flour/starch and water were adjusted to an average moisture content of 12%. They were mixed using a Kitchen-Aid Professional mixer (KPM5, KitchenAid, St. Joseph, Michigan, USA) for 8 min at speed 2. From each dough, 100 g (±0.05 g) were transferred to aluminum tins and left for fermentation for 90 min in a chamber at 30 °C with 90% of humidity. Half of the fermented dough was separated and prepared for volatile compounds analysis, which means the stop of the residual fermentation and final freezing prior to SHS analysis (Martínez-Anaya et al. 1990). The other half of the dough was baked at 190 °C for 40 min. After baking, the gluten-free breads were left at room temperature for 30 min and cut into loaves of 5 cm long. The crumb was separated from 1 cm to crust, to avoid the crumb contamination with crust volatile compounds (Birch et al. 2013). Finally, the crumbs were grounded and frozen at −20 °C in packages of 4 g prior to SHS-GC/MS analysis. Wheat bread dough and crumb samples were employed as control samples, since it is the most commonly consumed bread. In the wheat bread recipe there was no addition of HPMC, the rest of the ingredients and bread making conditions were identical. In order to understand the losses of fermentation volatile compounds during baking regarding the structure, the bread volumes were determined. Bread volumes were measured 24 h after baking, by duplicate (n = 2) using a laser sensor with the Volscan Profiler volume analyser (Stable Micro Systems, Surrey, UK).

Standards and solvents

To check the retention times and the m/z of the target ions, the following standards were purchased from Sigma Aldrich (Gillingham, UK): hexanal, 2-methylbutanol, 3-methylbutanol, 1-pentanol, 2-heptenal, hexanoic acid, acetaldehyde, 3-methylbutanal, 2,4-decadienal. Acetone and ethyl alcohol were supplied by Panreac (Barcelona, Spain).

Sample procedure: static headspace extraction (SHS)

The frozen samples (crumb and 90 min fermented dough) were tempered at room temperature during 30 min. Thereafter, 1 g (±0.050 g) of each sample (dough or crumb) was introduced in a 20 mL vial and sealed with a septum cap. After that, the sample was extracted for 90 min at 90 °C, without agitation, in a Static Headspace autosampler 7694 from Hewlett Packard (Palo Alto, California, USA). The loop and transfer line temperature were, respectively, 100 and 105 °C. The carrier gas employed was helium, supplied by Carburos Metálicos (Barcelona, Spain), with a carrier gas pressure of 23 psi and the vial pressurization was 14 psi for 0.2 min. The loop filling time was 0.2 min, the equilibration loop time was 0.05 min and the injection time 1 min. Each sample was analyzed in triplicate (n = 3).

Gas chromatography–mass spectrometry (GC–MS) conditions

GC–MS analyses were performed on a 7890A gas chromatograph coupled to a 5975C mass spectrometer detector (single quadrupole) equipped with a 7683B automatic injector and a Chemstation 5975C software, all from Hewlett Packard (Palo Alto, California, USA). Separation was achieved on a polar ZB-Wax column (100% polyethylene glycol, 60 m × 0.25 mm ID × 0.25 µm) obtained from Phenomenex (New South Wales, Australia). The GC was operated under programmed temperature conditions from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, afterwards temperature was increased to 114 °C (6.7 min) at 1 °C/min. The total run time was 30 min. The carrier gas was also helium, supplied by Carburos Metálicos (Barcelona, Spain), at a flow rate of 1.1 mL/min. The interface, ion source and quadrupole temperatures were 250, 230 and 150 °C, respectively. The MS scan parameters included a mass range of 15–350 m/z, operating in positive electron impact mode with ionization energy of 70 eV. Analyses were performed with selected ion monitoring mode (SIM), with one target (T) and two quantifier ions (Q_1 and Q_2) for each of the volatile compounds. The sixteen analytes were identified and confirmed by comparison of their retention times and mass spectra with standards and with the Mass Spectra Library (Wiley 7 N edition).

Data analysis

Principal component analysis (PCA) was calculated with the software Latentix (version 2.00, Latent5), with all GC/MS data autoscaled prior to the analysis. PCA is a suitable technique to describe major trends in a group of data and to detect possible outliers (Birch et al. 2013). The new variables (principal components) are constructed from a data matrix of the samples, where the scores are related to the samples (bread doughs and crumbs) and the loadings are related to the variables (volatile compounds). A large portion of the variability is often described by a few principal components. In this article, the scores plot and loadings plot are employed, showing the relationship between high/low values of the variables and the samples.

Results and discussion

Identification of volatile compounds in gluten-free bread doughs and crumbs

Sixteen main volatile compounds were identified both in dough and crumb in twelve breads by comparing with standards of their retention times and their target and two qualifier ions. As it is shown in Table 1, there were volatile compounds produced during fermentation and lipid oxidation and some of these by both (fermentation and lipid oxidation or fermentation and Maillard) (Pico et al. 2015). The study of the Maillard compounds in doughs and crumbs was not taken into consideration, since it was not possible to know if these were increased with the SHS temperatures. However, Maillard compounds are considered crucial only for the crust and the purpose of this study was to examine the influence of different flours and starches on the generation of volatile compounds in gluten-free doughs and crumbs.

Therefore, from the sixteen volatile compounds identified both in dough and crumb, acetone, ethyl alcohol, hexanal, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol, 2-heptenal, hexanoic acid and 2,4-decadienal were evaluated (in bold in Table 1).

Table 1 Main volatile compounds identified in all gluten-free bread doughs and crumbs

Volatile compounds	Source
Acetone	**Fermentation**
Ethyl alcohol	**Fermentation**
2-Methyl-1-butanol	**Fermentation**
3-Methyl-1-butanol	**Fermentation**
Hexanal	**Lipid oxidation**
1-Pentanol	**Lipid oxidation**
2-Heptenal	**Lipid oxidation**
2.4-Decadienal	**Lipid oxidation**
Hexanoic acid	**Fermentation & Lipid oxidation**
Acetaldehyde	Fermentation and Strecker degradation
3-Methylbutanal	Fermentation and Strecker degradation
2.3-Butanedione	Fermentation and Maillard
Acetoin	Fermentation and Maillard
Acetic acid	Fermentation and Maillard
Furfural	Fermentation and Maillard
Furfuryl alcohol	Fermentation and Maillard

Table 2 Fermentation and lipid oxidation volatile compounds identified in all doughs

	Acetone	Ethyl alcohol	Hexanal	2-Methyl-1-butanol	3-Methyl-1-butanol	1-Pentanol	2-Heptenal	Hexanoic acid	2.4-Decadienal
Wheat starch	3.56 ± 0.31	865.23 ± 105.12	27.12 ± 2.27	2.17 ± 0.20	4.14 ± 0.35	6.12 ± 0.72	2.39 ± 0.26	4.21 ± 0.28	2.31 ± 0.21
Corn starch	3.50 ± 0.25	488.82 ± 60.82	17.08 ± 1.07	2.03 ± 0.25	3.19 ± 0.21	2.75 ± 0.39	2.87 ± 0.29	0.00 ± 0.00	1.88 ± 0.16
Potato starch	3.46 ± 0.28	786.14 ± 87.39	4.67 ± 0.54	2.58 ± 0.31	4.10 ± 0.39	0.61 ± 0.04	0.41 ± 0.04	4.70 ± 0.36	3.74 ± 0.33
Wheat flour	4.37 ± 0.38	519.36 ± 74.25	9.23 ± 0.95	0.95 ± 0.13	1.76 ± 0.18	2.67 ± 0.39	0.57 ± 0.07	2.58 ± 0.19	1.90 ± 0.16
Yellow corn flour	12.71 ± 1.07	1070.17 ± 130.32	11.31 ± 1.15	3.84 ± 0.46	6.44 ± 0.52	2.29 ± 0.30	0.82 ± 0.09	4.53 ± 0.30	1.70 ± 0.12
White corn flour	18.83 ± 1.66	853.03 ± 101.72	18.28 ± 1.48	2.90 ± 0.31	5.27 ± 0.48	4.94 ± 0.61	0.70 ± 0.04	1.51 ± 0.12	0.78 ± 0.09
Buckwheat flour	14.41 ± 1.14	707.91 ± 85.03	1.02 ± 0.07	3.35 ± 0.35	5.48 ± 0.45	0.27 ± 0.04	0.15 ± 0.01	1.21 ± 0.07	0.98 ± 0.06
Rice flour	9.77 ± 0.89	905.88 ± 110.12	5.77 ± 0.59	3.42 ± 0.50	5.54 ± 0.41	0.98 ± 0.16	0.47 ± 0.06	4.87 ± 0.35	1.15 ± 0.15
Oat flour	8.11 ± 0.60	1024.37 ± 124.83	5.00 ± 0.37	3.77 ± 0.42	6.13 ± 0.56	1.98 ± 0.21	0.40 ± 0.02	3.72 ± 0.22	1.86 ± 0.14
Teff flour	19.16 ± 0.35	763.07 ± 87.55	1.34 ± 0.16	2.66 ± 0.28	4.38 ± 0.28	0.14 ± 0.09	0.29 ± 0.01	2.34 ± 0.26	1.64 ± 0.25
Quinoa flour	18.80 ± 1.49	607.07 ± 92.46	2.74 ± 0.15	3.24 ± 0.32	7.91 ± 0.32	0.37 ± 0.02	0.13 ± 0.05	1.29 ± 0.18	2.36 ± 0.13
Amaranth flour	13.08 ± 1.56	525.97 ± 75.13	1.69 ± 0.25	2.54 ± 0.37	3.87 ± 0.69	0.42 ± 0.06	0.20 ± 0.01	1.40 ± 0.07	0.75 ± 0.27

Results are given in area values ($\times 10^5$). Standard deviations (SD) are given after ± (n = 3)

Effect of the flour/starch on the different gluten-free bread doughs' volatile compounds

The results of nine fermentation and lipid oxidation compounds found in the doughs are given in area signal of the target ion (Table 2). Regarding these compounds, all twelve gluten free doughs present the same fermentation and lipid oxidation compounds, though in different concentration. Taking into consideration that only the kind of flour/starch was changed between the different doughs, keeping the rest of the recipe identical, this could led to conclude that the type of flour/starch affected the concentration of the fermentation and lipid oxidation compounds but not the creation/elimination of these (regarding the compounds detected with SHS-GC/MS, since only the most abundant compounds are present).

For an overview of the influence of the flour/starch on the generation of volatile compounds in the doughs, PCA was done for the data obtained (peak areas) (Fig. S1). Thus, it was possible to understand if the use of certain flour involved an increase or a decrease in the concentration of the volatile compounds that came from fermentation or lipid oxidation processes in relation to the other flours. The first principal component (PC1) explained 40.5% of the variability of the original variables. Regarding the scores plot, there was a clear separation between starches (negative x-axis) and flours (positive x-axis) and with regard to the loadings plot, the variables were clearly divided into lipid oxidation compounds (negative component of PC1) and fermentation compounds (positive component of PC1). Therefore, the separation of the gluten-free doughs between flours and starches could be attributed to the higher content in fermentation compounds of the flours and in lipid oxidation compounds in the case of starches. With respect to the Table 2, the measured contents of hexanal, 1-pentanol and 2-heptenal were especially higher in wheat and corn starch doughs than in flour doughs (except white corn flour). This could be surprising due to the contents of lipids in wheat and corn starch, which were reported to be lower than in flours, being in quinoa and amaranth flours the highest (as can be seen in Table S1, see supplementary data). However, not only the content of lipids should be taken into consideration. Lipoxygenase activity in flours also determines the amount of lipids that are susceptible to oxidation. With regard to starches, the most important involved enzymes have been amylases, glucoamylases and phosphorilases (BeMiller and Whistler 2009), but lipoxygenases have not been reported. It is supposed that the amount of lipoxygenases in starches caused by contamination of its isolation from flour could be negligible. Nevertheless, the main source of hydroperoxides decomposition that led to lipid oxidation compounds in storage food, like flour, were non-enzymatic reactions instead of enzymes ones (Gardner 1975). It implied a homolytic cleavage of the hydroperoxy group in a free-radical mechanism promoted by heat, photolysis, metal ions and other agents that promote free-radicals (Gardner 1975). Therefore, although lipoxygenase activity is not expected in starches, non-enzymatic lipid oxidation processes may have led to a higher concentration of lipid oxidation volatile compounds, such as hexanal, related to flours. In addition, cereals, pseudocereals and the corresponding flours contained a significant amount of antioxidants, like vitamin E and flavonoids, which trapped the hydroperoxides diminishing the final oxidation rate of the lipid oxidation. The amount of vitamin E (Alvarez-Jubete et al. 2010) and flavonoids (Hirose et al. 2010) has been reported to be much higher in pseudocereals, such as quinoa, than in cereals (Laus et al. 2012). However, antioxidant substances have not been reported in starches. Therefore, as the storage time of the flours/starches is also crucial (Maraschin et al. 2008), a balance between the lipid oxidation processes and the action of antioxidants should be considered to understand their content in volatile compounds from lipid oxidation processes.

Regarding the second principal component (PC2), it explains 26.7% of the variability. In the case of starches, wheat starch appears in the positive component of PC2 meanwhile corn starch in the negative axis of PC2. This means that in wheat starch there is a higher amount of volatile compounds from lipid oxidation than in corn starch. This is in concordance with those reported by Blaszczak et al. (2003), who found that wheat starch contains the highest content of total lipids, followed by corn starch and potato starch.

Taking into account the PC2 for gluten free flours, there is a clear separation between common gluten-free cereal flours (positive component of PC2) and pseudocereal flours (negative component of PC2). However, although teff belongs to the cereal crops family such as corn, rice and oat, it is in the same area of pseudocereals. It may be related to the chemical composition of teff flour, since the whole grain was milled as in pseudocereal flours, while the other flours were white flours. Their separation in the PC2 may be associated to the higher content of ethanol (fermentation marker) in corn, rice and oat doughs. As saccharose was added in the gluten-free breads, free sugars may not be the only limiting factor. Elgeti et al. (2014) reported that the α-glucosidase activity in quinoa flour was higher than in corn, rice or wheat flour. The release of fermentable sugars during fermentation could be partially attributed to the action of α-glucosidase and therefore the content of ethanol was higher in quinoa or amaranth dough. However, it has also been reported that flavonoids could act as inhibitors of α-glucosidase activity (Giménez-Bastida and Zieliński 2015; Li et al. 2009). The content of antioxidants, such as flavonoids, has been reported much higher in pseudocereals (Hirose et al. 2010) than in cereals like wheat (Giménez-Bastida and Zieliński 2015). This could explain the lower content in ethanol measured in these pseudocereals dough related to corn, rice and oat dough (Table 2). However, in the case of 3-methyl-1-butanol and 2-methyl-1-butanol, important markers of fermentation, the differences between cereal flours and pseudocereal flours were not so large, being the lowest in wheat flour. These compounds are Ehrlich alcohols generated during fermentation from leucine and isoleucine amino acids, respectively. Mota et al. (2016) reported that the average content in leucine and isoleucine is higher in quinoa than in crops like rice, and this could justify the lower differences in 3-methyl-1-butanol and 2-methyl-1-butanol measured between cereals and pseudocereals doughs (except wheat flour).

Related to the control sample, wheat flour dough is located in the negative component of PC1 and PC2 (high proportion of lipid oxidation volatile compounds). When the dough is made, due to the oxygenation during kneading, the content of vitamin E may have reduced, achieving higher lipid oxidation activities. This decrease has been reported to be higher (Alvarez-Jubete et al. 2010; Leenhardt et al. 2006) in wheat bread (47.6%) than rice bread (30.1%) and pseudocereals bread (7.5% in quinoa, 12.3% in buckwheat).

Effect of the flour/starch on the different gluten-free bread crumbs' volatile compounds

In Table 3 the results of the nine fermentation and lipid oxidation compounds found in the crumbs are given in area

Table 3 Fermentation and lipid oxidation volatile compounds identified in all crumbs

	Acetone	Ethyl alcohol	Hexanal	2-Methyl-1-butanol	3-Methyl-1-butanol	1-Pentanol	2-Heptenal	Hexanoic acid	2.4-Decadienal
Wheat starch	8.05 ± 0.63	116.64 ± 14.41	13.52 ± 1.21	0.04 ± 0.005	0.16 ± 0.01	3.28 ± 0.34	0.98 ± 0.09	5.38 ± 0.33	4.12 ± 0.44
Corn starch	5.93 ± 0.44	87.56 ± 10.77	11.54 ± 1.08	0.52 ± 0.060	0.97 ± 0.07	1.63 ± 0.15	2.03 ± 0.18	0.00 ± 0.00	3.14 ± 0.35
Potato starch	7.97 ± 0.71	102.38 ± 12.60	15.58 ± 1.39	0.02 ± 0.003	0.08 ± 0.01	3.20 ± 0.31	1.15 ± 0.13	5.42 ± 0.33	4.34 ± 0.41
Wheat flour	13.74 ± 1.12	211.07 ± 26.12	10.34 ± 0.91	0.03 ± 0.003	0.14 ± 0.01	3.98 ± 0.46	0.98 ± 0.09	2.38 ± 0.17	7.20 ± 0.70
Yellow corn flour	18.15 ± 1.51	330.23 ± 40.82	25.13 ± 2.33	0.03 ± 0.002	0.18 ± 0.01	7.64 ± 0.98	1.82 ± 0.13	5.07 ± 0.32	10.78 ± 1.08
White corn flour	45.18 ± 3.68	283.75 ± 35.17	74.39 ± 6.89	0.06 ± 0.005	0.44 ± 0.04	19.04 ± 2.23	2.21 ± 0.26	1.76 ± 0.15	5.18 ± 0.52
Buckwheat flour	29.58 ± 2.39	82.38 ± 10.17	1.57 ± 0.11	0.03 ± 0.004	0.10 ± 0.01	0.74 ± 0.07	0.18 ± 0.02	1.09 ± 0.08	0.99 ± 0.09
Rice flour	13.33 ± 1.12	177.98 ± 21.93	20.38 ± 1.86	0.01 ± 0.001	0.08 ± 0.01	4.30 ± 0.55	1.51 ± 0.17	5.31 ± 0.35	8.49 ± 0.86
Oat flour	15.63 ± 1.24	296.99 ± 36.70	9.74 ± 0.82	0.18 ± 0.024	0.52 ± 0.05	4.76 ± 0.56	1.03 ± 0.10	1.97 ± 0.14	8.26 ± 0.82
Teff flour	39.35 ± 1.47	263.72 ± 27.62	1.79 ± 0.75	0.20 ± 0.012	0.53 ± 0.03	0.69 ± 0.52	0.26 ± 0.09	1.77 ± 0.16	1.24 ± 0.76
Quinoa flour	56.27 ± 3.26	263.31 ± 32.61	1.43 ± 0.13	0.55 ± 0.025	1.53 ± 0.04	0.67 ± 0.09	0.20 ± 0.02	1.73 ± 0.13	0.71 ± 0.15
Amaranth flour	27.47 ± 4.63	324.05 ± 32.51	9.52 ± 0.15	0.54 ± 0.067	1.13 ± 0.13	4.93 ± 0.08	1.24 ± 0.01	1.45 ± 0.12	3.47 ± 0.08

Results are given in area values ($\times 10^5$). Standard deviations (SD) are given after ± ($n = 3$)

signal of the target ion to make comparisons. Taking into consideration these compounds, as was previously the case of the doughs, all twelve gluten-free bread crumbs presented the same fermentation and lipid oxidation compounds, but in different concentrations. This was in concordance with those reported by Dall'Asta et al. (2013), who studied the addition of chestnut flour to wheat breads. They found that, although wheat flour was very poor in volatile compounds compared to chestnut flour, wheat bread volatile profile was qualitatively comparable to those obtained for a supplement with chestnut flour, although in different amounts.

With the aim of interpreting the influence of the flour/starch on the generation of volatile compounds in crumbs, another PCA plot was generated (Fig. S2) from the obtained data (peak areas). There is a clear separation between pseudocereals (positive axis) and cereals (negative axis) regarding PC1 of the scores plot, which explains 41.4% of the variability of the original variables. Regarding the loadings plot, lipid oxidation compounds are located in the negative PC1 and fermentation compounds in the positive PC1. Therefore, cereal crumbs presented higher contents in lipid oxidation volatile compounds and pseudocereal crumbs in fermentation volatile compounds (mainly in 3-methyl-1-butanol and 2-methyl-1-butanol).

In general, there is a considerable reduction on the concentration of fermentation volatile compounds in all breads from dough to crumb due to their evaporation during baking. The rate of flavor compounds released depend not only on the volatility of the compound but also on the resistance to mass transfer from the matrix to the air. This resistance to mass transfer has been reported to depend on the macro- and microstructure and texture (Piazza et al. 2008). Therefore, the higher proportion of fermentation compounds measured in quinoa and amaranth crumbs due to a lower evaporation during baking (regarding the data of Tables 2 and 3) may be related with the bread structure. Since pseudocereals flours were wholemeal flours, the bran particles usually puncture and break the gas bubbles decreasing the volume of bread (Hager et al. 2012), leading to more compact breads. In order to understand the losses of volatile compounds during baking regarding the structure, bread volumes were measured and the results are shown in Table 4. In concordance with Hager et al. (2012), pseudocereal breads were much more compact than those of starches, which explain higher releases of ethanol in starches caused by an easier heat penetration. As can be seen in Table 4, the average volume of pseudocereal breads is 3.4 times lower than the average volume of starch breads. Taking into consideration the bread volumes and, therefore, the resistance to mass transfer in compact breads, the tendency of the % losses of ethanol in crumb is logical: starches > rice > oat and corns

Table 4 Percentage of ethanol evaporation from dough to crumb during baking and average bread volumes after baking (n = 2)

Bread	% Evaporation	Volume (cm^3)
Buckwheat flour	88.4	454.0
Potato starch	87.0	1271.0
Wheat starch	86.5	1539.0
Corn starch	82.1	1462.0
Rice flour	80.4	944.5
Oat flour	71.0	406.5
Yellow corn flour	69.1	497.5
White corn flour	66.7	469.0
Teff flour	65.4	425.0
Wheat flour	54.4	603.0
Quinoa flour	56.6	455.5
Amaranth flour	38.4	353.0

flours > teff and pseudocereal flours (Table 4). Thus, the lowest losses of ethanol are present in quinoa and amaranth, justifying their higher content in crumb in fermentation compounds.

The higher content of volatile compounds from lipid oxidation in cereal crumbs, especially in both corn flours, may be related with the balance between the content of lipids (which has not been reported the highest, see Table S1), the lipoxygenase activity and the lipid oxidation inhibitors (vitamin E and flavonoids), as it was explained in 'Effect of the flour/starch on the different gluten-free bread doughs' volatile compounds' section. A possible hypothesis to justify the highest content of lipid oxidation volatile compounds measured in yellow and white corn crumbs and rice crumb (Table 3) may be contributed to their high lipoxygenase activity values, as it has been reported by López-Duarte and Vidal-Quintanar (2009), Maraschin et al. (2008) and Zhang et al. (2009). Wheat and oat flours have also been reported to have lipoxygenase activity (Lampi et al. 2015; Leenhardt et al. 2006), wheat had lower activity than rice (Muñoz et al. 2015), which was in concordance with the result of each crumb in the scores plot (Fig. S2). In fact, oat flour lipoxygenase activity may be much higher, but oat grain may have been heat-treated to inactivate lipoxygenase (Lampi et al. 2015). However, the lipoxygenase activity in quinoa seeds has been reported to be low (Caussette et al. 1997). In addition, as it was explained, the antioxidant activity reported in pseudocereals implies a decrease in lipid oxidation volatile compounds.

It is important to point out that, regarding the PC2, quinoa and amaranth bread crumbs are located in the opposite side of buckwheat. The lower concentration of fermentation volatile compounds in buckwheat should be the cause of the distance between buckwheat and quinoa/amaranth. As it was indicated before, buckwheat presents the highest percentages of losses of fermentation volatile compounds, which could be somewhat related to its bread structure.

Combined results of gluten-free bread doughs and crumbs: the effect of the fermentation and lipid oxidation processes

From dough to crumb there were only differences in the amount of volatile compounds, but no creation nor removal of volatile compounds was observed, related to the SHS-GC/MS analyses. Maillard volatile compounds formed with high temperatures in crust and transferred to crumb, like 2-acetyl-1-pyrroline or 2,5-dimethyl-4-hydroxy-3(2H)-furanone, were not detected by SHS-GC/MS since they are at trace concentration in crumb. These justify the lack of generation of new volatile compounds from dough to crumb. Regarding the Tables 2 and 3, it could be concluded that, in general, doughs showed a higher proportion of fermentation volatile compounds and crumbs a higher proportion of lipid oxidation volatile compounds. This could be explained with the increase in the lipoxygenase action when the yeast activity decreases (Poinot et al. 2009), due to the oxygen necessity of lipoxygenases. Thus, when the rate of fermentation decreases, the lipoxygenase activity increases and, above all, with the elevated temperatures applied during baking the hydroperoxides are decomposed to lipid oxidation volatile compounds.

Towards the selection of the most suitable flour/starch in gluten-free bread aroma quality

Regarding Table 3 (crumb is the final product) and as far as it has been reported in literature, impact aroma compounds with a pleasant fruity perception (positive correlation) have been 3-methyl-1-butanol and 2-methyl-1-butanol and those reported as off-flavors (negative correlation) have been hexanal (grass) and 2,4-decadienal (fatty) (Pico et al. 2015). Quinoa crumb presented the highest content in 3-methyl-1-butanol and 2-methyl-1-butanol and also the lowest content in hexanal and 2,4-decadienal. However, quinoa contains between 0.1 and 5% of saponins (Valencia-Chamorro 2003), which are glycoside compounds that impart a bitter taste (Jancurová et al. 2009), masking the good perception of other compounds. Amaranth showed similar characteristics to quinoa, containing similar content of 3-methyl-1-butanol and 2-methyl-1-butanol but higher content of hexanal and 2,4-decadienal. Moreover, although in smaller amounts than in quinoa, it also contains bitter taste saponins (Oleszek et al. 1999). The improvements of the methods for saponins removal, without significant modifications of nutritive values, were observed (Jancurová et al. 2009). The third crumb containing high amounts of 2-methyl-1-butanol and 3-methyl-1-butanol was corn starch, with 6.5 and 36.9% less than in quinoa, respectively. The content of hexanal and 2,4-decadienal was almost 10 times and 5 times higher, respectively. However, it does not contain saponins, avoiding the bitter taste. Therefore, corn starch could to be a good option as a base of gluten-free bread in relation to its aroma quality. However, quinoa has been reported as containing high nutritional values in terms of protein, lipids, carbohydrates, vitamins, minerals and fiber (Alvarez-Jubete et al. 2010; Caussette et al. 1997; Hirose et al. 2010; Jancurová et al. 2009), nutritional values that are going to be lower with corn starch. Therefore, a mixture of them seems to be a suitable option.

Conclusion

Yellow and white corn, rice, oat, teff, buckwheat, amaranth and quinoa flours and wheat, corn and potato starches have been employed to compare the volatile profile of their doughs and crumbs. Volatile compounds from fermentation and lipid oxidation in the dough were similar but vary in concentrations. Higher concentrations of fermentation volatile compounds in the doughs and lipid oxidation volatile compounds in the crumbs were observed. Among the different gluten-free doughs and crumbs, the main volatile compounds were also the same, concluding that the type of flour/starch only affected the volatile compounds' concentration from fermentation and lipid oxidation processes (regarding SHS-GC/MS). Quinoa and amaranth crumbs presented the highest content of 2-methylbutanol and 3-methylbutanol (pleasant fruity aromas) but the lowest content of hexanal and 2,4-decadienal (grass and fatty off-flavors, respectively), which would indicated option to improve the gluten-free bread aroma, although their saponins imparted bitter taste. Corn starch was the next with higher content in 2-methylbutanol and 3-methylbutanol but lower content in hexanal and 2,4-decadienal in crumb, but the nutrition values were lower than in pseudocereals. Therefore, the proper mixture of quinoa flour with corn starch seems to be a suitable alternative towards an improved aroma in gluten-free breads.

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Comparison of the volatile profiles of the crumb of gluten-free breads by DHE-GC/MS

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ABSTRACT

The aroma of gluten-free bread has been considered of lower quality than that of the common wheat bread. With the aim of improving the aroma of gluten-free bread, the volatile profiles of the crumb of gluten-free breads made from rice, teff, buckwheat, amaranth and quinoa flours as well as corn starch, respectively, were evaluated. Wheat bread was used as a reference and dynamic headspace extraction together with GC/MS was employed. It was found that the whole grain breads, made with teff, quinoa and amaranth flours, presented a stronger aroma with higher number of important aroma contributors. Rice bread was characterised by the highest levels in nonanal and 2,4-decadienal and corn starch bread by 2,3-pentanedione and 2-furaldehyde. Teff presented the highest abundance of ethyl hexanoate and ethyl nonanoate, but also of alcohols and aldehydes from lipid oxidation. Quinoa and amaranth were classified by the highest content in Strecker and Ehrlich aldehydes as well as 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol or 3-hydroxy-2-butanone from fermentation. Corn starch bread was the closest to wheat bread in the PCA due to the highest content mainly in 2,3-butanedione and furfural as well as the lowest contents in 1-propanol, 1-hexanol and pentanal.

1. Introduction

The choice of the flour basis undoubtedly affects the final aroma of bread, one of the main characteristics perceived by the consumer. This is even more important in the case of gluten-free breads, since they are known to present a poor aroma quality. There is some information regarding the nutritional values (Alvarez-Jubete et al., 2010), rheology of the dough (Pruska-Kędzior et al., 2008), the texture, the volume and colour (Mancebo et al., 2015) or the staling (Ahlborn et al., 2005). However, there is little knowledge regarding gluten-free bread aroma as well as possible ways to improve it. For the moment, scarce information has been reported regarding the origin of the volatile compounds in gluten-free bread compared to wheat bread (Poinot et al., 2009) or the improvement of gluten-free bread aroma based on the addition of sugar-amino acid pairs (Pacyński et al., 2015). Moreover, the reported articles refer to a mixture of gluten-free (Poinot et al., 2009) or to a commercial preparation based on starches (Pacyński et al., 2015). To our knowledge, there is no report about the individual influence of different gluten-free flours and starches on the final aroma of the corresponding bread.

Gluten-free flours and starches present different contents in proteins, lipids or sugars, which are precursors that strongly determine the generation of the volatile compounds. Concretely, pseudocereals (quinoa, amaranth and buckwheat) have been reported to contain high nutritional values in terms of proteins, lipids, carbohydrates, vitamins, minerals and fiber (Alvarez-Jubete et al., 2010; Hirose et al., 2010). However, not only the level of lipids should be taken into consideration, but also the lipoxygenase and the antioxidant activities, the three factors influencing the final rate of the lipid oxidation reactions. The lipoxygenase activity has been considered to be significant in rice (Wongdechsarekul and Kongkiattikajorn, 2010), while in quinoa it has been considered to be negligible (Caussette et al., 1997); the antioxidant activity in pseudocereals, especially in quinoa, has been described to be higher than in cereals (Laus et al., 2012). The enzymes also influence the production of aroma compounds (Martínez-Anaya, 1996):

Abbreviations: DHE, (dynamic headspace); (GC/MS), gas chromatography-mass spectrometry; HPMC, (hydroxyl propyl methyl cellulose); MSD, (mass spectrometric detector); PCA, (principal component analysis); OT, (odour thresholds); PC1, (first principal component); PC2, (second principal component).

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(i) activity of proteases lead to free amino acids that can participate in Maillard reactions and in the Ehrlich pathway; (ii) activity of lipases lead to free fatty acids from lipids that are used by the lipoxygenases in the lipid oxidation process, resulting in a large number of volatile compounds; (iii) activity of α-glucosidases and amylases from the flours provide the yeast with free sugars like glucose and fructose, encouraging the fermentation.

The purpose of this research has been to investigate the influence of different gluten-free flours (rice, teff, buckwheat, amaranth and quinoa) and starches (corn) on the generation of volatile compounds in the crumb of the corresponding bread, as well as to compare the volatile profile with the wheat bread crumb. Understanding the influence of the flour basis could be essential to produce gluten-free breads with an improved aroma. The analyses were made by dynamic headspace extraction (DHE) followed by the determination by gas chromatography – mass spectrometry (GC/MS). The use of an inert gas to sweep the volatile compounds from the headspace to a Tenax-TA trap allows the concentration of the analytes and a decrease in the limits of detection. This makes DHE-GC/MS suitable for the comparison of volatile profiles between different gluten-free bread crumbs.

2. Materials and methods

2.1. Recipe ingredients

Corn starch and buckwheat flour were supplied by Biogan (Lystrup, Denmark), rice flour by Quaker (Chicago, USA), quinoa and amaranth flours from Aurion (Hjørring, Denmark) and teff flour from Holmen Crisp (Kapp, Norway). Wheat flour used for reference bread was purchased from Munkemølle (Odense, Denmark). Hydroxyl propyl methyl cellulose (HPMC) Methocel™ K4M was kindly provided by Dow Chemicals (Michigan, USA). Malteserkors dry baker's yeast (*Saccharomyces cerevisiae*) was purchased from Lallemand, De Danske Gærfabrikker (Grenå, Denmark); all yeasts belonging to the same batch to decrease the risk of different cell count of yeast and different contaminant bacteria. Salt, sugar and sunflower oil were purchased from the Danish local market and tap water was employed.

2.2. Bread making

The following ingredients, as % on flour or starch basis, were used in all the formulas: sunflower oil (6%), sucrose (5%), salt (1.8%), instant yeast (3%), HPMC (2%) and water (100%). The doughs were elaborated with a basis of 450 g (±0.05 g) of each flour or starch, adjusting to a final dry matter content of 88%. The ingredients were mixed for 10 min using a bread-mixing machine Fovea XBM 5 (Randers, Denmark), followed by 5 min of resting, mixed again for 10 min and finally rested another 5 min. The kneaded doughs were transferred to baking trays and left for fermentation for 90 min at 29 °C in a Termaks series 6000 cooling incubator (Bergen, Norway). Dough samples were then baked in a combi-steamer Conmatic line from Hounö (Randers, Denmark) for 25 min at 190 °C, without steam. After baking, the gluten-free breads were left at room temperature for 30 min, wrapped in aluminum foil and frozen at −18 °C in plastic bags, approved for food contact, until their analysis during the same week as preparation. Each bread was prepared in duplicate ($n = 2$).

Wheat bread was employed as control sample, since it is the most commonly consumed bread. In the wheat bread recipe there was no addition of HPMC, the rest of the ingredients and bread making conditions were identical.

2.3. Dynamic headspace extraction (DHE) of bread crumb

The frozen breads were thawed at room temperature for 30 min and slices of 5 cm were cut from the mid part of the bread to get the crumb for analysis. The crust of the two central slices was removed to a depth of 1 cm and discarded. Thereafter, the crumb was split in 4–6 small pieces and 15 g (±0.050 g) of each sample crumb was introduced in a 500 mL glass flask (7.5 cm in diameter). The DHE procedure was the same as Birch et al. (2013) work, only with the extraction of 60 min and in duplicate ($n = 2$).

2.4. Desorption and gas chromatography – mass spectrometry (GC-MS) conditions

The trapped volatiles were desorbed following Birch et al. (2013) procedure. The only change was the GC programme: from 30 °C (10min) to 240 °C (5min) at 8 °C/min. Volatile compounds were identified by probability based matching, higher than 90%, of their mass spectra with those of the database Wiley275.L (HP product no. G1035A). The identification was verified by the Kovats retention indices calculated using n-alkanes standards from C_5 to C_{22} (Hewlett Packard, Avondale, Pennsylvania). The Kovats retention indices, the target ions as well as the organoleptic characteristics of the 71 volatile compounds common for all the different bread crumbs and the 34 volatile compounds found only in some of the bread crumbs are shown in Table 1. The chromatogram of the corn starch bread crumb is given in Fig. 1 as an example.

2.5. Data analysis

In order to assess the variation of the volatile compounds between the different crumb samples, multivariate data analysis was conducted with the peak areas obtained by DHE-GC/MS, as the average of the two replicates of each bread analysed by duplicate ($n = 4$). Principal component analysis (PCA) was calculated with the software LatentiX version 2.00 (Latent5, Copenhagen, Denmark), with all the data auto-scaled prior to the analysis.

The One-Way Anova of the volatile compounds in the different breads was performed with the software Statgraphics Centurion version XVII (Statpoint Technologies, Warrenton, Virginia).

3. Results and discussion

3.1. Influence of the flour/starch on the volatile profile of the gluten-free bread crumbs

Breads made with corn starch and five gluten-free flours (rice, teff, quinoa, amaranth and buckwheat) were tested in order to evaluate the effect of the flour basis on the final aroma of gluten-free bread. A total of 105 volatile compounds were detected among the different breads (Table 1), 71 of them in common for all the bread crumbs (Table 2).

In order to better understand these differences, the PCA of the 105 volatile compounds was calculated (Fig. 2). Regarding the scores plot of the samples, there is a clear separation between the breads coming from whole grain flours (quinoa, amaranth and teff) in the positive component of the PC1 and white flour basis (rice, wheat and corn starch) in the negative component of the PC1. Additionally, the bread crumbs from cereal grains (rice, teff and corn starch) situated in the positive component of the PC2 were clearly distinguished from pseudocereals (quinoa and amaranth) situated in the negative component of the PC2. Buckwheat bread has not been taken into consideration for this discussion since it

Table 1
List of volatile compounds found among the seven bread crumbs (corn starch and rice, teff, quinoa, amaranth, buckwheat and wheat bread crumbs) by DHE-GC/MS. The Kovats retention indices were calculated comparing the retention times with those of the serie of n-alkanes from C_5 to C_{22}.

Volatile compounds	Target ion	Kovats RI Calculated	Kovats RI [a,b] Literature	Organoleptic [a,b,c,d] characteristics
Hexane	57	600	600	Gasoline-like
Octane	43	783	800	Gasoline-like
2-Methylpropanal	72	804	802	Malty
Ehtyl acetate	43	879	878	Fruity
2-Butanone	43	894	894	Acetone-like
2-Methylbutanal	41	905	905	Fruity, almond
3-Methylbutanal	41	909	910	Apple-like
Benzene	78	925	926	Petroleum-like
2,2,4,6,6-Pentamethyl-heptane	57	935	943	Metallic
2-Ethylfuran	81	940	944	Musty, earthy
Ethyl propanoate	57	947	947	Pineapple-like
Ethyl isobutyrate	43	956	955	Fruity, strawberry
Pentanal	44	966	967	Pungent
2,3-Butanedione	43	970	970	Buttery
Decane	57	988	1000	Fruity, sweet
α -pinene	93	1008	1008	Pine
Toluene	91	1025	1025	Benzene-like
Ethyl butyrate	71	1030	1029	Pineapple-like
1-Propanol	31	1042	1042	Alcohol-like
Camphene	93	1047	1047	Green-minty notes
2,3-Pentanedione	43	1059	1054	Caramel, fruity
Hexanal	56	1078	1077	Green grass
2-Beta-pinene	93	1092	1092	Pine
2-Methyl-1-propanol	43	1100	1100	Wine, malty
Sabinene	93	1111	1133	Citrus, spicy
Verbenene	91	1114	976	Spicy, minty
Ethylbenzene	91	1118	1118	Pungent
Isoamyl acetate	43	1125	1125	Banana-like
1-Methoxy-2-propanol	45	1131	1131	Sweet ether-like
2-Butylfuran	81	1135	1138	Wine, sweet
Ethyl valerate	88	1139	1147	Fruity, orange
Delta-3-carene	93	1142	1144	Turpentine-like
1-Butanol	56	1156	1156	Alcoholic, banana
β-Mircene	41	1165	1166	Pleasant, terpene
Cumene	105	1168	1168	Gasoline-like
1-Penten-3-ol	57	1170	1169	Buttery,milky
α -terpinene	121	1173	1174	Gasoline-like
Cyclopentanone	55	1174	1176	Petroleum-like
o-Xylene	91	1176	1175	Sweet
2-Heptanone	43	1181	1181	Fruity
Heptanal	70	1183	1183	Fatty, pungent
Limonene	68	1189	1194	Citrus
Dodecane	57	1196	1200	Minty
3-Methyl-1-butanol	55	1215	1215	Balsamic, alcohol
2-Pentylfuran	81	1236	1236	Floral, fruit
Ethyl hexanoate	88	1241	1241	Fruity, juicy
2-Methyl-thiazole	58	1242	1241	Green
Gamma-Terpinene	93	1247	1247	Citrus, herbal
Styrene	104	1256	1256	Sweet, floral
3-Methyl-3-buten-1-ol	41	1258	1258	Sweet, fruity
1-Pentanol	55	1263	1263	Fusel-like
Cymene	119	1271	1271	Sweet
Hexyl acetate	43	1281	1281	Fruity, sweet
α -terpinolene	93	1285	1285	Sweet-pine
3-hydroxy-2-butanone	45	1288	1288	Buttery
Octanal	41	1294	1294	Strong, fruity
1-Hydroxy-2-propanone	43	1302	1303	Sweet,burnt
2-Ethyl-1-butanol	43	1312	1312	Wine, onion
4-Methyl-1-pentanol	56	1325	1325	Nutty
2-Heptenal	41	1327	1328	Green, fatty
3-Methyl-2-buten-1-ol	71	1331	1334	Green, fruity
α -methylstyrene	118	1333	1334	Sharp- aromatic
Ethyl heptanoate	88	1339	1338	Pineapple, fruity
6-Methyl-5-Hepten-2-one	43	1341	1341	Fatty, green, citrus
1-Hexanol	56	1360	1360	Sweet alcohol
3-Hexen-1-ol	67	1386	1386	Grassy-green
Nonanal	57	1391	1391	Waxy, green, fatty
3-Ethyl-2-methyl-1,3-(Z)-hexadiene	67	1412	1421	Pungent
3-Furaldehyde	95	1425	1426	Almond
2-Octenal	55	1428	1425	Green, fatty
Ethyl octanoate	88	1433	1433	Fruity, floral

Table 1 (*continued*)

Volatile compounds	Target ion	Kovats RI Calculated	Kovats RI [a,b] Literature	Organoleptic [a,b,c,d] characteristics
Acetic acid	43	1446	1445	Vinegar-like
1-Octen-3-ol	57	1450	1450	Mushroom
1-Heptanol	70	1457	1457	Fatty
2-Furaldehyde	96	1459	1458	Almond-like
6-Methyl-5-Hepten-2-ol	95	1463	1464	Mushroom, earthy
2-Ethyl-1-Hexanol	57	1489	1489	Sweet, floral
2-Acetylfuran	95	1501	1501	Balsamic, cinammon
Pyrrole	67	1510	1511	Chloroform-like
Benzaldehyde	105	1519	1519	Bitter almond
Ethyl nonanoate	88	1533	1533	Fruity, rose
1-Octanol	56	1557	1557	Orange-rose

Volatile compounds	Target ion	Kovats RI Calculated	Kovats RI [a,b] Literature	Organoleptic [a,b,c,d] characteristics
Isobutyric acid	43	1562	1561	Butter, fat, cheesy
Pinocarvone	81	1570	1569	Sweet, herbal
Bornyl acetate	95	1581	1595	Pine, camphor
Hexadecane	57	1585	1600	Fruity, sweet
Calarene	161	1592	1591	Green, earthy
Butyrolactone	42	1627	1626	Sweet, caramel
Ethyl decanoate	88	1634	1633	Grape, fruity
Benzeneacetaldehyde	91	1638	1638	Honey, floral
Acetophenone	105	1650	1650	Sweet-acacia
p-Allylanisole	148	1667	1666	Anise
2-Hydroxy-benzaldehyde	122	1677	1677	Bitter almond
Verbenone	107	1715	1712	Minty, spicy
Beta-bisabolene	69	1723	1723	Herbal
Azulene	128	1741	1746	Chamomile
α-dimethyl-α-benzenemethanol	121	1756	1759	Sweet, earthy
α -Cumyl alcohol	43	1756	1759	Hyacinth, lilac
2,4-Decadienal	81	1810	1820	Fatty, deep-fried
Trans-anethole	148	1825	1824	Anise oil
Butoxyethoxyethyl acetate	87	1845	1838	Fruity
Benzyl alcohol	79	1875	1875	Fruity, balsamic
Phenylethyl alcohol	91	1912	1912	Rose-like
Phenol	94	1997	1997	Acrid
Indole	117	2387	2398	Jasmine

[a] https://pubchem.ncbi.nlm.nih.gov/compound/.

[b] http://www.pherobase.com.

[c] http://www.thegoodscentscompany.com.

[d] Birch et al. (2013).

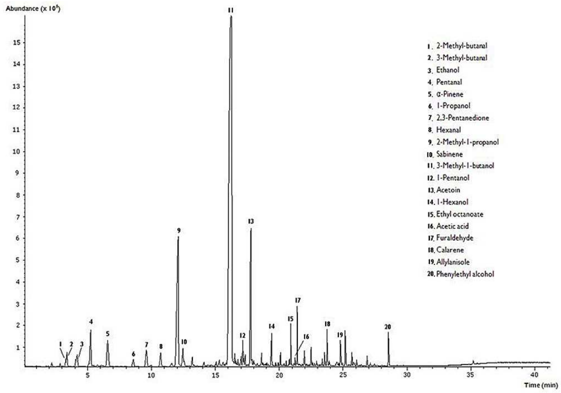

Fig. 1. Chromatogram of the volatile compounds detected in the corn starch bread crumb in SCAN mode. Only the most abundant compounds were numbered.

was located near the coordinate origin. Wheat bread employed as a reference was located really near the x-axis regarding the PC2 but in a quadrant separated from the rest of gluten-free breads.

Taking into consideration the loadings plot of the volatile compounds, 77 of the 105 volatile compounds were situated in the positive component of the PC1, most of them important for the aroma of bread (Birch et al., 2014; Pico et al., 2015). In the positive PC1 and PC2, alcohols and aldehydes from the lipids oxidation, esters, some terpenes and benzenic compounds were included, where teff crumb is located. Meanwhile, Strecker and Ehrlich aldehydes, like 2-methypropanal or 3-methylbutanal, as well as alcohols from fermentation, like 1-propanol, 2-methyl-1-propanol or 3-methyl-1-butanol, and 3-hydroxy-2-butanone were located in the positive PC1/negative component of PC2, in the same area than quinoa and amaranth crumbs. This means that the aroma of the whole grain breads (quinoa, amaranth and teff) probably is stronger than the breads made from white flours or starch, which could be justified by the content of macro constituents of the flours. In the whole grain flours the bran, the germ and the endosperm are milled while in the white flours only the endosperm is grounded, resulting in higher content of proteins and lipids in the case of whole grain flours. For example, whole-grain wheat flour has been reported with 13.21% of proteins and 2.50% of lipids, while wheat white flour with 10.33% of proteins and 0.98% of lipids (USDA Nutrient Database, 2009). The proteins can be degraded to amino acids which are the main precursors of the Maillard reactions, the Strecker degradation and the Ehrlich pathway, while the lipids are the main precursors of the volatile compounds from the lipids oxidation (Birch et al., 2014; Pico et al., 2015). Moreover, most of the enzymes are localised in the aleurone layer of the bran and in the germ, enzymes such as amylases, proteases, lipoxygenases or lipases that are of utmost importance in the generation of precursors for volatile compounds (Martínez-Anaya, 1996). Finally, the amount

Table 2
Results of the volatile compounds, in peak area of the target ion (x 10^7), found in the seven bread crumbs by DHE-GC/MS (corn starch and rice, teff, quinoa, amaranth, buckwheat and wheat bread crumbs). Standard deviations (SD) are given after ± (n = 4). Different letters in the same row indicate significant differences in the One-way Anova (significant level 95%). The volatile numbers corresponds to the numbering of the PCA (Fig. 2).

Volatile compound	Number	Wheat flour	Corn starch	Rice flour	Teff flour	Quinoa flour	Amaranth flour	Buckwheat flour
Hexane	1	0.10 a ± 0.01	0.13 a ± 0.02	0.16 a ± 0.01	0.67 b ± 0.09	0.71 bc ± 0.09	0.80 c ± 0.02	0.09 a ± 0.01
Octane	2	0.18 a ± 0.01	0.20 ab ± 0.02	0.60 d ± 0.03	1.07 e ± 0.07	0.58 d ± 0.07	0.26 b ± 0.05	0.38 c ± 0.03
2-Methylpropanal	3	0.22 b ± 0.03	0.15 a ± 0.02	0.23 b ± 0.03	0.34 c ± 0.02	0.34 c ± 0.03	0.75 d ± 0.02	0.39 c ± 0.05
Ehtyl acetate	4	11.58 b ± 1.37	2.56 a ± 0.03	2.48 a ± 0.30	20.21 d ± 1.24	13.24 bc ± 0.03	27.93 e ± 2.39	16.20 c ± 2.33
2-Butanone	5	0.66 de ± 0.0004	0.54 bc ± 0.004	0.64 cd ± 0.06	0.37 a ± 0.04	0.77 ef ± 0.06	0.78 f ± 0.01	0.53 b ± 0.09
2-Methylbutanal	6	0.96 a ± 0.01	1.51 b ± 0.05	1.86 c ± 0.11	3.96 e ± 0.02	2.49 d ± 0.02	4.41 f ± 0.22	2.35 d ± 0.30
3-Methylbutanal	7	1.61 a ± 0.07	2.87 b ± 0.48	3.08 b ± 0.12	4.47 c ± 0.38	5.71 d ± 0.41	3.42 b ± 0.26	3.39 b ± 0.45
Benzene	8	0.12 ab ± 0.01	0.12 bc ± 0.01	0.13 c ± 0.001	0.13 bc ± 0.01	0.13 c ± 0.002	0.13 bc ± 0.01	0.11 a ± 0.01
Ethyl propanoate	9	0.72 b ± 0.09	0.16 a ± 0.01	0.15 a ± 0.003	2.75 e ± 0.13	1.23 c ± 0.03	1.45 c ± 0.22	1.87 d ± 0.05
Pentanal	10	0.91 a ± 0.12	1.02 ab ± 0.12	1.49 b ± 0.13	3.57 d ± 0.49	2.58 c ± 0.28	2.14 c ± 0.24	0.74 a ± 0.09
2,3-Butanedione	11	55.16 d ± 3.75	49.52 c ± 1.48	23.78 a ± 1.85	43.09 bc ± 2.10	40.84 b ± 5.03	28.62 a ± 2.91	27.83 a ± 4.73
α -pinene	12	31.68 bc ± 2.45	25.94 b ± 1.96	59.44 d ± 8.08	30.60 b ± 1.98	37.95 c ± 4.47	14.39 a ± 0.94	26.57 b ± 1.33
Toluene	13	0.78 a ± 0.003	0.82 a ± 0.01	0.71 a ± 0.04	2.99 d ± 0.38	2.42 c ± 0.10	1.74 b ± 0.24	2.56 c ± 0.02
1-Propanol	14	16.52 cd ± 1.38	9.70 a ± 0.48	9.75 a ± 1.10	14.57 bc ± 1.80	17.34 d ± 0.70	16.63 cd ± 1.22	14.30 b ± 1.97
Camphene	15	0.53 c ± 0.04	0.42 b ± 0.04	0.66 d ± 0.02	0.46 bc ± 0.03	0.61 d ± 0.06	0.28 a ± 0.04	0.41 b ± 0.02
2,3-Pentanedione	16	7.84 c ± 0.97	19.46 d ± 1.85	2.74 a ± 0.29	7.79 c ± 0.54	4.65 b ± 0.56	2.53 a ± 0.33	1.79 a ± 0.06
Hexanal	17	8.79 a ± 0.63	5.54 a ± 0.64	8.32 a ± 1.33	50.54 c ± 6.31	17.07 b ± 0.44	15.86 b ± 1.50	4.94 a ± 0.83
2-Beta-pinene	18	3.08 b ± 0.33	2.78 b ± 0.06	3.87 d ± 0.45	3.61 cd ± 0.22	3.65 d ± 0.26	1.31 a ± 0.20	3.14 bc ± 0.27
2-Methyl-1-propanol	19	102.74 c ± 5.87	85.50 b ± 2.63	50.41 a ± 2.65	84.23 b ± 7.76	109.60 c ± 0.80	104.83 c ± 5.17	106.50 c ± 8.81
Sabinene	20	11.79 bc ± 1.45	9.47 b ± 0.89	11.63 bc ± 1.05	16.25 d ± 2.02	12.26 c ± 1.22	5.30 a ± 0.71	15.07 d ± 0.33
Verbenene	21	1.71 d ± 0.06	1.39 c ± 0.02	1.33 c ± 0.10	0.46 a ± 0.02	0.62 b ± 0.09	0.58 ab ± 0.01	0.69 b ± 0.07
Ethylbenzene	22	0.08 a ± 0.01	0.09 a ± 0.01	0.07 a ± 0.01	0.35 d ± 0.04	0.15 b ± 0.02	0.15 b ± 0.003	0.26 c ± 0.03
1-Methoxy-2-propanol	23	0.61 a ± 0.04	8.19 e ± 0.39	5.42 d ± 0.35	3.83 b ± 0.13	4.73 c ± 0.57	4.37 bc ± 0.52	4.61 c ± 0.46
Delta-3-carene	24	0.08 a ± 0.003	0.10 a ± 0.0001	0.68 b ± 0.09	0.37 ab ± 0.03	4.43 c ± 0.54	0.39 ab ± 0.04	0.58 b ± 0.03
1-Butanol	25	1.43 c ± 0.16	1.11 b ± 0.06	0.78 a ± 0.06	2.02 e ± 0.21	2.08 e ± 0.02	1.80 de ± 0.15	1.56 cd ± 0.20
α -terpinene	26	0.33 b ± 0.03	0.35 bc ± 0.01	0.33 b ± 0.03	0.49 d ± 0.05	0.32 b ± 0.04	0.16 a ± 0.02	0.38 c ± 0.01
o-Xylene	27	0.11 a ± 0.01	0.17 bc ± 0.02	0.14 ab ± 0.002	0.55 e ± 0.04	0.33 d ± 0.001	0.19 c ± 0.03	0.34 d ± 0.02
2-Heptanone	28	0.36 a ± 0.02	0.32 a ± 0.12	0.74 b ± 0.11	3.41 e ± 0.36	1.53 d ± 0.0005	1.25 cd ± 0.16	1.04 c ± 0.05
Heptanal	29	0.89 c ± 0.04	0.65 b ± 0.12	0.51 a ± 0.07	2.24 e ± 0.06	1.04 d ± 0.05	0.64 b ± 0.08	0.43 a ± 0.04
Limonene	30	1.18 ± 0.07	1.35 ± 0.11	1.36 ± 0.07	1.88 ± 0.16	3.02 ± 0.29	1.72 ± 0.21	2.00 ± 0.06
3-Methyl-1-butanol	31	149.00 c ± 8.53	112.40 ab ± 19.51	87.80 a ± 3.38	145.25 bc ± 4.30	154.62 c ± 0.23	164.12 c ± 1.46	146.63 c ± 4.73
2-Pentylfuran	32	3.34 a ± 0.21	3.67 a ± 0.62	9.05 b ± 1.78	25.97 c ± 1.31	9.63 b ± 0.79	3.58 a ± 0.41	3.25 a ± 0.38
Ethyl hexanoate	33	0.59 a ± 0.07	0.46 a ± 0.05	0.54 a ± 0.05	9.65 c ± 0.10	0.53 a ± 0.004	0.73 b ± 0.12	0.54 a ± 0.04
Gamma-Terpinene	34	0.71 b ± 0.05	0.70 b ± 0.18	0.69 b ± 0.03	1.07 d ± 0.07	0.75 bc ± 0.02	0.48 a ± 0.05	0.89 c ± 0.03
Styrene	35	3.06 a ± 0.27	1.76 ± 0.09	1.42 ± 0.13	27.99 ± 1.43	5.60 ± 0.23	5.89 ± 0.51	32.81 ± 0.35
3-Methyl-3-buten-1-ol	36	1.18 b ± 0.05	1.06 b ± 0.06	0.79 a ± 0.02	1.47 c ± 0.01	1.51 c ± 0.20	1.51 c ± 0.20	1.51 c ± 0.14
1-Pentanol	37	3.20 a ± 0.29	3.92 a ± 0.54	6.27 b ± 0.39	17.03 d ± 0.19	8.12 c ± 0.58	6.21 b ± 0.49	3.56 a ± 0.25
Cymene	38	4.56 b ± 0.46	4.23 b ± 1.03	3.65 ± 0.35	5.59 c ± 0.69	4.41 b ± 0.51	2.61 a ± 0.24	4.65 bc ± 0.31
α -terpinolene	39	0.13 ab ± 0.01	0.17 bc ± 0.01	0.17 bc ± 0.01	0.24 c ± 0.02	0.49 e ± 0.05	0.12 a ± 0.01	0.41 d ± 0.03
3-hydroxy-2-butanone	40	61.03 c ± 3.31	46.32 a ± 5.73	55.55 b ± 2.22	56.72 b ± 1.31	78.60 d ± 2.28	64.61 c ± 0.94	49.56 a ± 2.31
1-Hydroxy-2-propanone	41	1.09 ab ± 0.08	1.21 bc ± 0.44	1.18 bc ± 0.06	1.14 b ± 0.13	3.09 d ± 0.29	1.56 c ± 0.24	0.59 a ± 0.02
α -methylstyrene	42	0.06 ab ± 0.005	0.04 a ± 0.01	0.11 c ± 0.01	0.17 d ± 0.02	0.39 e ± 0.0001	0.15 d ± 0.03	0.08 bc ± 0.01
6-Methyl-5-Hepten-2-one	43	0.11 a ± 0.01	0.17 a ± 0.003	0.11 a ± 0.01	0.34 b ± 0.04	1.08 d ± 0.05	0.66 c ± 0.06	0.30 b ± 0.04
1-Hexanol	44	15.56 b ± 0.50	6.91 a ± 0.91	24.59 c ± 4.85	39.44 e ± 0.76	30.47 d ± 0.69	24.78 c ± 1.06	16.99 b ± 0.84
Nonanal	45	0.70 a ± 0.07	1.71 d ± 0.14	3.58 f ± 0.18	2.18 e ± 0.21	1.21 c ± 0.04	0.74 a ± 0.05	0.96 b ± 0.07
3-Ethyl-2-methyl-1,3-(Z)-hexadiene	46	0.33 c ± 0.02	0.51 d ± 0.15	0.33 c ± 0.05	0.62 e ± 0.06	0.46 d ± 0.04	0.21 b ± 0.01	0.13 a ± 0.01
Ethyl octanoate	47	3.44 b ± 0.13	6.47 c ± 1.09	2.39 a ± 0.29	7.08 c ± 0.72	2.10 a ± 0.01	2.45 ab ± 0.03	2.90 ab ± 0.03
Acetic acid	48	0.42 a ± 0.01	0.14 a ± 0.02	0.21 a ± 0.04	0.30 a ±0.004	2.79 b ± 1.35	0.32 a ± 0.02	0.36 a ± 0.04
1-Octen-3-ol	49	0.12 a ± 0.01	1.42 c ± 0.36	1.69 c ± 0.22	5.34 e ± 0.40	2.75 d ± 0.10	0.89 b ± 0.03	0.63 b ± 0.02
1-Heptanol	50	0.39 a ± 0.01	0.50 b ± 0.06	0.49 b ± 0.06	1.33 e ± 0.05	1.20 d ± 0.06	0.65 c ± 0.06	0.45 ab ± 0.04
2-Furaldehyde	51	6.06 d ± 0.34	6.72 d ± 1.17	1.55 a ± 0.28	4.43 c ± 0.22	4.63 c ± 0.04	2.55 b ± 0.26	2.05 ab ± 0.13
2-Acetylfuran	52	0.58 d ± 0.06	0.71 e ± 0.01	0.38 c ± 0.03	0.29 b ± 0.002	0.32 bc ± 0.03	0.17 a ± 0.004	0.24 ab ± 0.046
Pyrrole	53	0.36 c ± 0.03	0.13 a ± 0.01	0.16 a ± 0.004	1.19 d ± 0.17	0.32 bc ± 0.05	0.13 a ± 0.01	0.22 ab ± 0.01
Benzaldehyde	54	9.32 f ± 0.34	2.25 a ± 0.01	3.24 bc ± 0.21	2.79 ab ± 0.07	3.67 c ± 0.16	7.06 e ± 0.51	5.92 d ± 0.76
Ethyl nonanoate	55	0.08 a ± 0.01	0.04 a ± 0.01	0.27 c ± 0.02	1.11 d ± 0.10	0.17 bc ± 0.001	0.11 ab ± 0.003	0.05 a ± 0.003
1-Octanol	56	0.09 a ± 0.005	0.10 a ± 0.01	0.36 cd ± 0.04	0.34 c ± 0.03	0.38 d ± 0.02	0.27 b ± 0.02	0.12 a ± 0.01
Bornyl acetate	57	1.23 d ± 0.02	1.20 d ± 0.12	1.37 e ± 0.08	1.05 c ± 0.03	0.81 b ± 0.02	0.65 a ± 0.05	1.00 c ± 0.06
Calarene	58	4.81 bc ± 0.11	6.10 d ± 1.15	5.20 c ± 0.27	4.32 b ± 0.19	3.07 a ± 0.11	3.11 a ± 0.15	4.34 b ± 0.28
Butyrolactone	59	0.05 ab ± 0.01	0.03 a ± 0.002	0.04 ab ± 0.01	0.22 ± 0.03	0.11 d ± 0.000001	0.08 c ± 0.01	0.06 b ± 0.01
Ethyl decanoate	60	0.15 a ± 0.01	0.27 b ± 0.03	0.36 c ± 0.03	0.26 b ± 0.03	0.29 ± 0.002	0.36 c ± 0.01	0.26 b ± 0.02
Benzeneacetaldehyde	61	1.90 c ± 0.11	2.08 c ± 0.29	1.52 b ± 0.16	4.53 e ± 0.16	1.24 ab ± 0.09	1.13 a ± 0.09	3.46 d ± 0.23
Acetophenone	62	0.14 a ± 0.01	0.44 de ± 0.08	1.44 f ± 0.03	0.45 e ± 0.05	0.37 cd ± 0.05	0.24 b ± 0.03	0.35 c ± 0.03
Verbenone	63	1.26 c ± 0.03	1.41 d ± 0.17	1.40 d ± 0.06	1.05 b ± 0.06	0.94 a ± 0.01	0.90 a ± 0.03	0.96 ab ± 0.05
Beta-bisabolene	64	0.20 c ± 0.01	0.24 d ± 0.02	0.24 d ± 0.01	0.35 e ± 0.01	0.13 a ± 0.004	0.13 a ± 0.002	0.16 b ± 0.01
Azulene	65	0.10 a ± 0.01	0.13 b ± 0.02	0.22 c ± 0.01	0.71 e ± 0.02	0.36 d ± 0.01	0.24 c ± 0.02	0.22 c ± 0.01

Table 2 (*continued*)

Volatile compound	Number	Wheat flour	Corn starch	Rice flour	Teff flour	Quinoa flour	Amaranth flour	Buckwheat flour
α-dimethyl-α-benzenemethanol	66	0.05 a ± 0.00	0.07 ab ± 0.01	0.16 bc ± 0.02	0.25 cd ± 0.06	0.30 d ± 0.06	0.60 e ± 0.09	0.07 ab ± 0.01
2,4-Decadienal	67	0.30 b ± 0.03	0.87 d ± 0.02	1.74 e ± 0.15	0.70 c ± 0.07	0.76 cd ± 0.10	0.09 a ± 0.01	0.05 a ± 0.01
Benzyl alcohol	68	0.07 a ± 0.01	0.18 b ± 0.02	0.11 a ± 0.001	0.22 bc ± 0.03	0.35 d ± 0.04	0.19 bc ± 0.03	0.22 c ± 0.01
Phenylethyl alcohol	69	11.86 c ± 0.62	13.17 c ± 1.26	10.92 bc ± 2.14	7.43 a ± 0.22	11.65 bc ± 1.11	8.98 ab ± 0.65	18.65 d ± 2.39
Phenol	70	0.46 d ± 0.01	0.22 c ± 0.02	0.60 e ± 0.04	0.14 a ± 0.01	0.14 a ± 0.004	0.17 b ± 0.01	0.16 ab ± 0.01
Indole	71	0.53 a ± 0.03	1.99 d ± 0.22	2.70 e ± 0.18	1.11 c ± 0.16	0.89 b ± 0.08	0.58 a ± 0.06	1.23 c ± 0.10

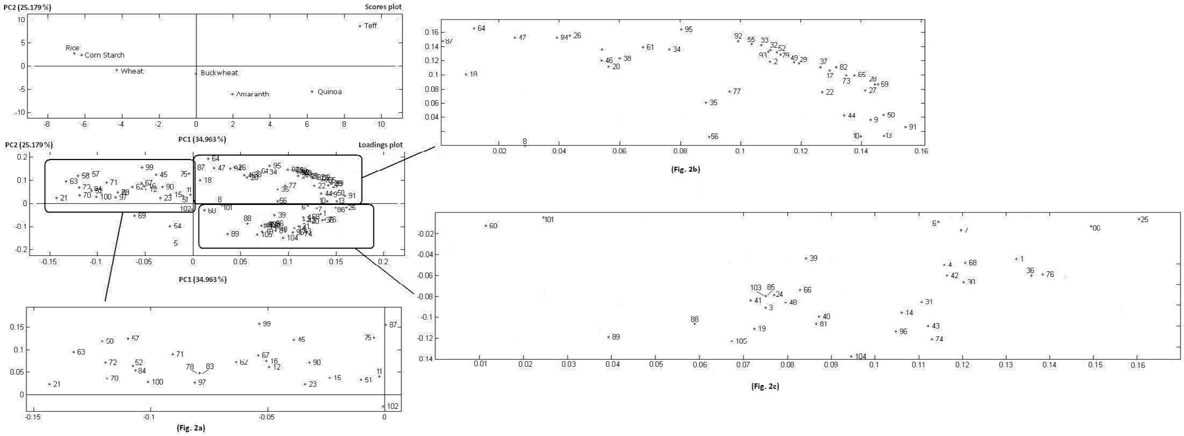

Fig. 2. PCA of the gluten-free breads and wheat bread analysed by DHE-GC/MS. The scores plot represents the seven samples and the loadings plot the 105 volatile compounds found among all the breads. The numbers corresponding to each volatile compound are indicated in Table 2 (common volatile compounds for the seven breads) and 3 (non-common volatile compounds for the seven breads). The zoom of those zones that are difficult to distinguish are depicted in Fig. 2a, b and c.

of the antioxidants flavonoids is also decisive in the concentration of volatile compounds from lipids oxidation and this has been reported to be significant in teff, quinoa and amaranth flours (Inglett et al., 2015). The higher amount of proteins and lipids together with the higher occurrence of enzymes in the bran and germ (Belitz, Grosch, & Schieberle) that have been removed in white flours justify the greater abundance of volatile compounds in whole grain breads.

Regarding the 34 volatile compounds that were non-common for all the gluten-free breads, since they were not present or were under the limits of detection (Table 3), only a few of them have been previously reported in wheat bread. 2-heptenal and 2-octenal have been considered as minor volatile compounds in wheat bread crumb (Ruiz et al., 2003), arising mainly from lipid oxidation reactions (Poinot et al., 2010) with green and fatty odour characteristics, while hexyl acetate has been reported in wheat bread with fruity and sweet notes (Paraskevopoulou et al., 2012). 3-Furaldehyde from Maillard reactions (Poinot et al., 2010), 1-Penten-3-ol from the lipoxygenase activity (Gassenmeier and Schieberle, 1995) and decane have been considered minor volatiles in wheat bread (Ruiz et al., 2003). However, only isoamyl acetate, isobutyric acid formed by fermentation (Ruiz et al., 2003) and octanal by lipid oxidation (Birch et al., 2013) have been reported as important contributors to wheat bread crumb aroma with positive correlation with the final aroma of bread. The banana-like isoamyl acetate was found in all the breads except bread made from rice flour while the cheesy-like isobutyric acid was only found in rice bread. However, although 2-octenal, octanal, hexyl acetate and isobutyric acid have been reported in the literature of wheat bread, they have not been found in the wheat bread crumb elaborated as a control sample.

The rest of non-common volatile compounds have not been reported with an important impact in the final aroma of bread. Among them, 2-butylfuran, β-mircene, cyclopentanone have been only present in rice bread crumb, while 2-methyl-thiazole, isobutyric acid and α -cumyl alcohol have been only present in quinoa bread crumb.

3.2. Volatile compounds common for all the studied gluten-free bread crumbs

From now on, only the 71 volatile compounds common for the seven breads are taking into consideration for the discussion of the PCA (Fig. 2, numbers assigned in Tables 2 and 3), focusing in those reported in the literature as important aroma contributors (Birch et al., 2014; Pico et al., 2015).

The volatile compounds found in the area of rice and corn starch bread (negative PC1/positive PC2) with the highest content were nonanal and 2,4-decadienal (rice) as well as 2,3-pentanedione and 2-furaldehyde (corn starch). The content of lipids has been reported to be generally higher in the pseudocereals, quinoa and amaranth, than in cereals like wheat (Alvarez-Jubete et al., 2009). Therefore, the volatile compounds coming from the lipid oxidation process as nonanal and 2,4-decadienal, were expected to be higher in content in quinoa and amaranth breads. However, not only the level of lipids should be taken into consideration, but also the lipoxygenase and the antioxidant activities. The lipoxygenase activity has been considered to be important in cereals like stored rice (Wongdechsarekul and Kongkiattikajorn, 2010) and wheat (Leenhardt et al., 2006) and on the contrary, lipoxygenase activity in quinoa has been considered to be negligible (Caussette et al., 1997), which justify the supporting of the lipid oxidation

Table 3
Volatile compounds found only in some of the gluten-free bread crumbs by DHE-GC/MS. The empty box means that the volatile compound was not detected. The volatile numbers corresponds to the numbering of the PCA (Fig. 2).

Volatile compounds	Number	Wheat	Quinoa	Rice	Corn starch	Amaranth	Teff	Buckwheat
2,2,4,6,6-Pentamethyl-heptane	72	✓		✓	✓	✓	✓	✓
2-Ethylfuran	73	✓	✓	✓		✓	✓	
Ethyl isobutyrate	74	✓	✓			✓	✓	✓
Decane	75	✓			✓		✓	
Ethyl butyrate	76	✓	✓			✓	✓	✓
Isoamyl acetate	77	✓	✓		✓	✓	✓	✓
2-Butylfuran	78			✓				
Ethyl valerate	79					✓	✓	✓
β-Mircene	80			✓				
Cumene	81		✓	✓		✓	✓	✓
1-Penten-3-ol	82	✓	✓	✓			✓	✓
Cyclopentanone	83			✓				
Dodecane	84			✓	✓			
2-Methyl-thiazole	85		✓					
Hexyl acetate	86		✓	✓		✓	✓	✓
Octanal	87			✓	✓	✓	✓	✓
2-Ethyl-1-butanol	88	✓	✓		✓	✓	✓	✓
4-Methyl-1-pentanol	89		✓			✓		
2-Heptenal	90	✓	✓	✓	✓		✓	✓
3-Methyl-2-buten-1-ol	91	✓	✓		✓	✓	✓	✓
Ethyl heptanoate	92	✓	✓	✓		✓	✓	
3-Hexen-1-ol	93	✓				✓	✓	
3-Furaldehyde	94	✓				✓	✓	
2-Octenal	95						✓	
6-Methyl-5-Hepten-2-ol	96	✓	✓			✓	✓	✓
2-Ethyl-1-Hexanol	97	✓	✓	✓	✓	✓		
Isobutyric acid	98		✓					
Pinocarvone	99	✓		✓	✓	✓	✓	✓
Hexadecane	100	✓		✓				
p-Allylanisole	101		✓		✓	✓	✓	✓
2-Hydroxy-benzaldehyde	102	✓						✓
α -Cumyl alcohol	103		✓					
Trans-anethole	104		✓	✓		✓	✓	✓
Butoxyethoxyethyl acetate	105	✓	✓	✓		✓	✓	
Total number of compounds		**19**	**21**	**19**	**14**	**21**	**23**	**17**

reactions in rice crumb. On the other hand, the antioxidant activity in pseudocereals, especially in quinoa, has been described to be higher than in cereals like wheat (Laus et al., 2012). Quinoa and amaranth have been considered excellent sources of vitamin E (Alvarez-Jubete et al., 2010), a strong antioxidant, much higher in quinoa and amaranth than in cereals like teff and wheat (Inglett et al., 2015). Both have also been recognized as good sources of flavonoids, mainly quercetin in quinoa (Hirose et al., 2010) and caffeic acid, ferulic acid and p-hydroxybenzoic acid in amaranth (Alvarez-Jubete et al., 2010). The lower lipoxygenase activity together with the higher antioxidant activity, inhibit the generation of volatile compounds from lipid oxidation, such as nonanal and 2,4-decadienal, in pseudocereal crumbs.

Finally, the content of 2,3-butanedione and 2-furaldehyde in corn starch were slightly higher than in the other gluten-free breads and really similar to that of wheat bread (One-way Anova, Table 2). In the case of 2,3-pentanedione, it was clearly higher than in the rest of breads. 2,3-Butanedione and 2,3-pentanedione have been reported as prominent ketones in wheat bread (Seitz, 1998), while 2-furaldehyde has been considered an important volatile compound for bread odour quality in sourdough wheat breads (Plessas et al., 2008). The three volatile compounds can be generated through Maillard reactions during baking (Pico et al., 2015), explaining why their concentrations are higher in crust than in crumb (Chiavaro et al., 2008).

Teff crumb, located in the positive PC1/positive PC2 of the PCA (Fig. 2), was characterised by the highest abundance of alcohols and aldehydes from lipid oxidation, esters, terpenes and benzenic compounds. As can be seen in Table 2, the esters ethyl propanoate, ethyl hexanoate, ethyl octanoate and ethyl nonanoate were higher in teff bread, all of them with fruity odours (Table 1). The content of ethyl octanoate was almost the same as in corn starch (One-way Anova, Table 2) and ethyl acetate and ethyl decanoate were higher in amaranth crumb. Ethyl hexanoate, ethyl octanoate, ethyl nonanoate as well as ethyl acetate have been described as the most aroma-active esters in wheat bread crumb (Birch et al., 2014).

On the other hand, the wealth of alcohols and aldehydes from lipid oxidation was clearly higher in teff bread than in the rest of breads, being for 1-octen-3-ol, hexanal or 1-pentanol, pentanal 44.5, 5.8, 5.3 and 3.9 times higher than in wheat crumb (Table 2). Although the content of unsaturated fatty acids has been reported lower in teff than in quinoa and amaranth grains, the content of vitamin E in teff has been reported to be 30 times lower than the content in quinoa and 15 times lower than the content in amaranth (Inglett et al., 2015). Then, the lipid oxidation processes were less inhibited in teff bread and the contents of volatile compounds from lipid oxidation were encouraged.

Quinoa and amaranth breads, placed in the positive PC1/negative PC2, were distinguished by the highest abundance in Strecker and Ehrlich aldehydes, alcohols from fermentation, 3-hydroxy-2-butanone and acetic acid. 3-Methylbutanal, 2-methylbutanal as well as 2-methylpropanal can be generated through the Ehrlich pathway during fermentation and also through the Strecker reactions that take place during baking as a Maillard step. In both cases, the amino acids are the common source for the generation of the aldehydes; 3-methylbutanal comes from leucince, 2-methylbutanal from isoleucine and 2-methypropanal from valine (Pico et al., 2015). In general, the content of proteins has been reported higher in the pseudocereals quinoa and amaranth than in wheat (Alvarez-Jubete et al., 2009). Also, the average content of leucine, isoleucine and valine in different varieties of quinoa and amaranth have been reported higher than in rice (Mota et al., 2016), explaining the highest abundance of these Strecker and Ehrlich aldehydes in quinoa and amaranth breads. On the other hand, 1-propanol, 2-methyl-1-propanol and 3-methyl-1-butanol were volatile compounds exclusively generated by fermentation while 3-hydroxy-2-butanone and acetic acid were mainly generated by fermentation (glycolysis and fatty acids pathway in the yeast cell, respectively) but could have also been generated through Maillard reactions during baking (Pico et al., 2015). The content of acetic acid in quinoa was clearly highest than in the rest of breads. However, the content of the fermentation volatile compounds 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 3-hydroxy-2-butanone in quinoa and amaranth breads was very similar to that of wheat bread and higher than rice and corn starch bread. This could be the reason of the separation in the negative PC2 between wheat and rice/corn starch breads, the three breads made with white-cereal flours. The higher abundance of volatile compounds from fermentation in quinoa could be explained by the higher α-glucosidase activity reported in quinoa compared to rice and wheat (Elgeti et al., 2014), leading to more free glucose units that are used as substrate by the yeast. Quinoa has also been characterised by a substantial amylase activity (Caussette et al., 1997).

Finally, the reference wheat bread was the only located in the negative PC1/negative PC2, separated then from all the gluten-free breads. It showed the highest content in phenylethyl alcohol (from fermentation and Maillard) and benzaldehyde (from fermentation, lipid oxidation and Maillard), both reported as important aroma-active compounds (Birch et al., 2014) and positively correlated with the final aroma of wheat bread (Pico et al., 2015). The content of phenylethyl alcohol was very similar to quinoa bread crumb and the content of benzaldehyde was considerably higher than in the rest of bread crumbs. The rest of volatile compounds were similar or normally higher in the gluten-free breads. The closest gluten-free bread to wheat bread was corn starch, due to the similar higher content in 2,3-butanedione, 2-furaldehyde and terpenes like verbenene as well as the similar lower content in 1-pentanol and pentanal.

3.3. *Selection of the most suitable gluten-free flour/starch regarding the volatile profile*

Nonanal and 2,4-decadienal were most abundant in rice bread compared to the other breads. They have been reported as very aroma-active compounds in wheat bread, with very low odour thresholds (OT) of 1 and 0.07 mg L^{-1} respectively (Birch et al., 2014). Although nonanal has been described as an odorant with pleasant aroma properties (Paraskevopoulou et al., 2012) due to its citrus notes, there are some controversies since it has also been characterised by fatty notes (Table 1). In turn, 2,4-decadienal has been clearly reported as an off-flavour volatile compound (Quílez et al., 2006) for its fatty-rancid attributes (Table 1). Likewise, in the case of teff bread, the alcohols and aldehydes from lipid oxidation have been reported as off-flavours in bread (Pico et al., 2015), with pungent-fatty notes (pentanal, heptanal and 1-heptanol), green-fatty notes (hexanal and 1-hexanol), fusel-like (1-pentanol) or mushroom-like (1-octen-3-ol) (Table 1), counteracting the pleasant odour of the esters. Moreover, among all the esters in teff bread, only ethyl hexanoate and ethyl octanoate have been described with low OTs, 1 and 92 $\mu g\ L^{-1}$ respectively (Birch et al., 2014). Almost all the alcohols and aldehydes from lipid oxidation found in teff bread also presented very low OTs, 12 $\mu g\ L^{-1}$ for pentanal, 4.5 $\mu g\ L^{-1}$ for hexanal, 3 $\mu g\ L^{-1}$ for heptanal, 1 $\mu g\ L^{-1}$ for 1-octen-3-ol and 3 $\mu g\ L^{-1}$ for 1-heptanol (Birch et al., 2014). Only 1-pentanol and 1-hexanol exhibited high OTs, 4000 and 2500 $\mu g\ L^{-1}$ respectively (Birch et al., 2014). Therefore, the uses of large amount of rice flour or teff flour are discouraged in order to improve the final aroma of gluten-free bread.

Regarding corn starch, it contains the highest amount of 2,3-butanedione, 2,3-pentanedione and 2-furaldehyde compared to the rest of gluten-free breads. The content of 2,3-butanedione and 2-furaldehyde was very similar to that of wheat bread while the content of 2,3-pentanedione was clearly higher than in the rest of breads. Both ketones present pleasant buttery notes (Table 1) and low OTs, being 15 $\mu g\ L^{-1}$ for 2,3-butanedione and 30 $\mu g\ L^{-1}$ for 2,3-pentanedione (Belitz et al., 2009). 2-furaldehyde has been positively correlated with the final aroma of bread due to its almond-sweet characteristics, although it has been reported with high OT (3000 $\mu g\ L^{-1}$).

The Strecker and Ehrlich aldehydes found in highest abundance in quinoa and amaranth breads have also been described with pleasant descriptors like fruity-almond (2-methylbutanal), apple-like (3-methylbutanal) or malty (2-methylpropanal) and low OTs of 4 $\mu g\ L^{-1}$, 0.2 $\mu g\ L^{-1}$ and 1 $\mu g\ L^{-1}$ (Belitz et al., 2009), respectively. However, the alcohols from fermentation, found in highest abundance in quinoa and amaranth breads and similar to wheat bread, did not present low OTs. They were 9000 $\mu g\ L^{-1}$ for 1-propanol, 3200 $\mu g\ L^{-1}$ for 2-methyl-1-propanol, 500 $\mu g\ L^{-1}$ for 1-butanol and 250 $\mu g\ L^{-1}$ for 3-methyl-1-butanol (Birch et al., 2014). Nevertheless, they have been reported with pleasant descriptors like alcoholic (1-propanol, 1-butanol), malty (2-methyl-1-propanol) or balsamic (3-methyl-1-butanol). Between all of them, 3-methyl-1-butanol and 2-methyl-1-propanol have been, by far, two of the most abundant volatile compounds in all the samples, as can be seen in Table 2 and Fig. 2. Finally, 3-hydroxy-2-butanone, highest in quinoa and amaranth breads and similar to wheat bread, has been described with a positive correlation with the final aroma of wheat bread due to its buttery notes (Table 1) but with a high OT of 800 $\mu g\ L^{-1}$ (Birch et al., 2014). Thus, although 3-hydroxy-2-butanone has been characterised as an important aroma compound in wheat bread with an odour activity value (OAV) lower than 0.1, it was not so aroma-active as 2,3-butanedione, the other key ketone in wheat bread (Birch et al., 2014).

Therefore, the similarities with wheat bread suggest a mixture of corn starch and quinoa/amaranth flours as a suitable option to improve the final aroma of gluten-free bread due to: (i) the high content of 2,3-butanedione and 2-furaldehyde for corn starch bread, which led to corn starch bread as the closest to wheat bread in the PCA; (ii) the high content of 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 3-hydroxy-2-butanone for quinoa and amaranth breads, which located wheat bread in the negative PC2; (iii) the positive correlation with the final aroma of bread for 2,3-butanedione, 2-furaldehyde, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 3-hydroxy-2-butanone; (iv) as well as the low contents in the off-flavours originated from the lipids oxidation for corn starch, quinoa and amaranth breads.

By all means, not only the aroma but also other factors should be taken into consideration, such as the nutritional values and the non-volatile compounds that give the taste. Quinoa contains between 0.1 and 5% of natural saponins depending on the variety (Valencia-Chamorro, 2003), which are glycoside compounds that impart a bitter taste (Jancurová et al., 2009), masking the good perception of other compounds. Amaranth, although in smaller amounts, also contains bitter taste saponins (Oleszek et al., 1999).

However, the improvements of the methods for the desaponification, without significant modifications of nutritive values, are nowadays encouraged (Jancurová et al., 2009). On the other hand, corn starch presents poor nutritional values and lacks proteins, and vitamins, as it is the starchy part of the endosperm. However, quinoa has been reported as containing high nutritional values in terms of protein, lipids, carbohydrates, vitamins, minerals and fiber (Alvarez-Jubete et al., 2010; Caussette et al., 1997; Hirose et al., 2010).

4. Conclusions

The use of six different gluten free flours and starches, and wheat flour as a reference, led to different aroma profiles of the corresponding bread crumbs, analysed by DHE-GC/MS. Most of the volatile compounds were common for all the gluten-free breads and wheat bread, including volatile compounds from fermentation, lipid oxidation and Maillard reactions and covering important volatile compounds reported in the literature. Among the non-common volatile compounds, only isoamyl acetate, isobutyric acid and octanal have been previously described as important contributors for wheat bread aroma. Regarding the common volatile compounds, whole grain breads (quinoa, amaranth and teff) showed similarities between them (placed in PC1) and differences to the white breads (placed in PC2). Rice was characterised by the highest content in the lipid oxidation products nonanal and 2,4-decadienal (low OTs and green-fatty and rancid-fatty notes, respectively) and corn starch in 2,3-pentanedione and 2-furaldehyde (low OT and caramel-like and high OT and almond-like, respectively). Teff was characterised by the highest abundance in alcohols and aldehydes from lipid oxidation, also with low OTs and reported as off-flavours in bread. Quinoa and amaranth were identified by the highest content in Strecker and Ehrlich aldehydes (low OTs, fruity and malty descriptors), alcohols from fermentation (high OTs but pleasant alcoholic and balsamic descriptors), 3-hydroxy-2-butanone and acetic acid (high OTs and buttery and vinegar-like, respectively). The high contents of 2,3-butanedione and 2-furaldehyde for corn starch bread as well as the high contents of 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 3-hydroxy-2-butanone for quinoa and amaranth breads were similar for wheat bread. Moreover, corn starch bread was the closest to wheat bread in the PCA. Therefore, the choice of the gluten-free flour is really important in order to improve the final aroma. With this background, the use of large amounts of rice and teff flours was discouraged, while the suitable proportion between quinoa/amaranth and corn starch was recommended.

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VIII. Section 4

Volatile compounds in gluten-free flours and starches and the importance of the aroma of gluten-free bread crust. Selection of the most suitable flour/ starch for the improvement of the gluten-free bread crust aroma

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Comparison of different extraction methodologies for the analysis of volatile compounds in gluten-free flours and corn starch by GC/QTOF

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A B S T R A C T

Gluten-free bakery products usually exhibit weak aroma. Their main constituents are flours and starches, which contain aroma precursors but can also contribute additional volatiles in low concentrations. Static headspace (SHS), solid-phase microextraction (SPME) and solvent-assisted flavour evaporation (SAFE) coupled to GC/QTOF were compared for their efficacy in the analysis of volatiles in corn starch. SPME-GC/QTOF was selected as the most suitable methodology based on the number of detected compounds, LODs, repeatability as well as simplicity. It was successfully applied for the quantification of volatiles in corn starch and qualitative comparison of different gluten-free flours. Hexanal, 3-methyl-1-butanol, 1-pentanol, 1-octen-3-ol, acetic acid, furfural, benzaldehyde, (*E*)-2-nonenal, phenylethyl alcohol and short-medium chain acids were found in all the flours and corn starch. Quinoa flour and corn starch showed the highest contents of pyrazines, terpenes and esters, while teff, buckwheat and rice flours presented the highest contents of 3/2-methyl-1-butanol, acetoin and organic acids.

1. Introduction

Gluten-free products, most notably bread, are characterised by a weak, poor aroma. One of the main ingredients of gluten-free products is the gluten-free flour or starch, which can affect the final aroma in two different ways. Flours/starches can be a source of precursors for volatile compounds, including lipids and antioxidants for lipid oxidation products, proteins for Strecker and Ehrlich aldehydes, sugars for volatile compounds from fermentation and amino acids and reducing sugars for Maillard reactions or even enzymes. Flours/starches can also provide volatile compounds themselves. Therefore, monitoring the volatile compounds from the very beginning (the flour as an ingredient) could help to understand how they evolve during product manufacturing. Moreover, examination of the volatile profile of the flours and starches could be employed as a quality control parameter for freshness, both in a flour mill or as a raw material.

Some research has already been conducted on the analysis of volatile compounds in flours, such as wheat flour (Czerny & Schieberle, 2002), rye flour (Kirchhoff & Schieberle, 2002), pea flour (Murat, Gourrat, Jerosch, & Cayot, 2012), extruded rice flour, soy flour (Vodovotz, Zasypkin, Lertsiriyothin, Lee, & Bourland, 2000) and even chestnut flour (Dall'Asta et al., 2013). Starches are considered tasteless and odourless, although commercial starches often have odour notes characteristic of their sources (Sayaslan, 2005). The aroma of cereal starches has been attributed to the autoxidation of lipids on the surface of the starch granule, with corn starch reported as the most flavourful (Sayaslan, 2005). Nevertheless, the volatile compounds present in wheat flour have been found to be of only minor importance to the aroma of bread (Cho & Peterson, 2010). In any case, there is currently no report about the volatile compounds of gluten-free flours (rice, corn, quinoa, teff, amaranth, buckwheat, etc) and their possible influence on gluten-free products.

Flours, and above all starches, have been reported to contain generally low concentrations of volatile compounds (Sayaslan, 2005). Therefore, analytical techniques with low limits of detection are required, such as MS/MS. Regarding the sample treatment, there have mainly been two options: (i) solvent extraction, using

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Abbreviations: CAR, carboxen; CCD, central composite design; D, desirability function; DVB, divinylbenzene; GC/QTOF, gas-chromatography/quadrupole-time of flight; LOD, limit of detection; LOQ, limit of quantification; OT, odour threshold; PC, principal component; PCA, principal component analysis; PDMS, polydimethylsiloxane; PFTE, polytetrafluoroethylene; RSD, relative standard deviation; SAFE, solvent assisted flavour evaporation; SHS, static headspace; SPME, solid-phase microextraction.

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solvent assisted flavour evaporation (SAFE) (Czerny & Schieberle, 2002; Kirchhoff & Schieberle, 2002; Murat et al., 2012); (ii) headspace extraction, using solid-phase microextraction (SPME) (Dall'Asta et al., 2013; Murat et al., 2012) or dynamic headspace extraction (DHE) (Sayaslan, 2005). SAFE extracts have reportedly been richer in high-molecular weight volatile compounds, while SPME extracts have been richer in low-molecular weight volatile compounds (Mayuoni-kirshinbaum, Tietel, Porat, & Ulrich, 2012), which is highly important for those compounds that are very volatile and co-elute with the solvent in the SAFE methodology (Majcher & Jeleń, 2009). SPME has usually been preferred because it is a quick, simple and solvent-free technique (Thompson-Witrick et al., 2015). On the contrary, SAFE has been reported to allow for a more complete isolation than SPME, thereby more accurately preserving the native profile of the food product (Roth et al., 2014).

Thus, the aim of this work has been to compare the analytical characteristics of three methodologies, in order to determine which is the most suitable for the analysis of volatile compounds in gluten-free flours and corn starch. SPME, static headspace (SHS) and SAFE together with GC/QTOF were evaluated. The three methodologies were examined using corn starch, since it is expected to present a lower concentration of volatile compounds than gluten-free flours and it is one of the most employed starches in the preparation of gluten-free bread. Subsequently, in order to check the suitability of the selected methodology, each was applied to the characterisation of different gluten-free flours. The examination of these gluten-free flours could potentially lead to future investigations of ways to improve the aroma of gluten-free products, due to the knowledge of the mechanisms of evolution of the volatile compounds from the flour to the final product.

2. Materials and methods

2.1. Materials, reagents and standards

For the solvent extraction methodology, diethyl ether was acquired from Panreac (Barcelona, Spain) and dry ice was obtained from Linde Group (Munich, Germany). For the analytical characterisation of the three methods, all the standards (sub-section 2.4) were purchased from Sigma-Aldrich (Steinheim, Germany); methanol was obtained from LAB-SCAN (Gliwice, Poland).

2.2. Description of samples

The development and characterisation of the SAFE methodology, SHS method as well as the SPME method were carried out with the corn starch "Maizena" purchased from Unilever (Barcelona, Spain). The selected methodology was applied to the aroma profile analysis of rice flour (Sarchio, Carpi, Italy), teff flour (Saluteff, San Martín del Valle, Spain), buckwheat flour (Nature & Cie, Vallet, France) and quinoa flour (Anapqui, La Paz, Bolivia).

2.3. Sample treatments

2.3.1. Solvent extraction: Solvent assisted flavour evaporation (SAFE)

Corn starch (50 ± 0.050 g) was inserted into a Soxhlet thimble (Prat Dumas, Couze-et-Saint-Front, France) and transferred to a Soxhlet apparatus. The extraction was carried out with 300 mL of diethyl ether for 8 h at 40 °C and then the distillation flask was cooled to 25 °C for 10 min. Afterwards, the extract was concentrated by means of a 50 cm Vigreux column until approximately 150 mL was obtained (for around 20 min) and then it was cooled to 25 °C for another 10 min. Then, the extract was distilled employing a SAFE apparatus (Baeng, Manching, Germany). The distillation flask, the central head and the legs were thermostated at 21 °C using a water recirculation thermostat (Huber, Edison, NJ). A vacuum pressure of 10^{-6} mbar was achieved by means of an HP 40 B2 from Vacuubrand (Wertheim, Germany). The cooling trap was filled with dry ice and the condensation flask was also immersed in a Dewar vessel full of dry ice. The extract was added by dropping 10 mL of sample every 2 min from the dropping funnel. The extract took 20 min to reach 25 °C and to collect the drops retained in the cooling trap. Later, the extract was concentrated again using the Vigreux column until a final volume of 1 mL was reached (for around 65 min at 38 °C). The extract was left again for 10 min to reach room temperature and was immediately injected into the GC/QTOF. A careful cleaning of the SAFE system between samples was necessary, as otherwise contamination was observed. First, the system was rinsed with acetone, in order to remove the remnants of dichloromethane and medium polar volatile compounds and then it was cleaned with water and soap in order to remove any remaining volatile compounds and fat. Finally, the system was abundantly rinsed with deionised water and dried with a small volume of acetone. Each sample was analysed in triplicate.

2.3.2. Static headspace (SHS)

Corn starch (3 g ± 0.050 g) was weighed into a 20-mL vial and sealed with a magnetic screw cap provided with polytetrafluoroethylene (PTFE)/silicone septa; 750 μL of internal standard (3,3-dimethyl-2-butanol/4-allylsyringol, 0.2 mg L^{-1} in dimethyl sulfide) were added. The sample extraction time and temperature were 30 min at 100 °C, respectively, with agitation. The syringe temperature was 110 °C. The gas phase was injected into the GC injector port at 270 °C, with an injection volume of 2 mL. Each sample was analysed in triplicate.

2.3.3. Solid-phase microextraction (SPME)

Corn starch (0.5 g ± 0.005 g) was weighed into a 20-mL vial and sealed with a magnetic screw cap provided with PTFE/silicone septa; 100 μL of internal standard (3,3-dimethyl-2-butanol/4-allyl syringol, 0.2 mg L^{-1} in deionised water) were added. The selected fibre was 50/30 μm DVB/CAR/PDMS (Sigma Aldrich, Gillingham, UK). The sample was incubated for 5 min at 50 °C (without the fibre) and then extracted for 51 min at 50 °C, without agitation. The fibre was inserted into the GC injector port for thermal desorption for 5 min at 270 °C. The fibre was conditioned for 30 min at 270 °C after each analysis. Each sample was analysed in triplicate.

2.4. GC/QTOF chromatographic conditions

GC/MS analyses were performed on a 7890 A gas chromatograph coupled to a 7200 quadrupole-time of flight (QTOF) mass spectrometer with MassHunter B.07.00 software, all from Agilent Technologies (Santa Clara, CA). The GC was equipped with a CombiPAL RSI 85 autosampler from CTC Analytics AG (Zwingen, Switzerland). The separation was achieved on a polar Innowax column (100% polyethylene glycol, 30 m × 0.25 mm ID × 0.25 μm) obtained from J&W Scientific (Agilent Technologies). The GC was operated under programmed temperature conditions: from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, then the temperature was increased to 114 °C (6.7 min) at 1 °C/min, afterwards it was increased to 136 °C (0 min) at 2.5 °C/min and finally it was increased to 245 °C (5 min) at 85 °C/min. The total run time was 43 min. The carrier gas was helium, supplied by Carburos Metálicos (Barcelona, Spain), at a flow rate of 1.1 mL/min. The injector temperature was 270 °C, working in splitless mode. The interface, ion source and quadrupole temperatures were 250 °C, 230 °C and 150 °C, respectively. Analyses were performed in scan mode over a mass range of m/z 20–350, in electron ionisation mode with energy of 70 eV. All 73 volatile compounds shown in Table 1 were identified by their mass spectrum (NIST MS Search 2.2 & MS Inter-

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Table 1
Volatile compounds identified in corn starch with the evaluated methodologies: Solvent extraction (SAFE), Static Headspace (SHS) and Solid-phase microextraction (SPME). The target ions (T), the retention time in min (t_R) as well as the Kovats Index (KI) calculated and of the .literature for each compound are also provided.

volatile compound	T	t_R	KI calculated	KI literature	SHS	SPME	SAFE
1-propanol	55.0542	4.455	889	988	*		
hexanal	56.0620	5.104	1060	1080	*	*	*
3-heptanone	57.0340	6.743	1073	1160	*	*	*
1-methylpyrrole	81.0569	6.952	1077	1140	*	*	
2-heptanone	43.0183	7.829	1148	1175		*	
heptanal	70.0771	7.912	1154	1151	*	*	
eucalyptol	81.0704	8.361	1191	1193	*	*	*
limonene	68.0614	8.568	1202	1202		*	*
pyrazine	80.0367	8.883	1211	1216	*	*	*
2-pentylfuran	81.0329	9.875	1237	1235	*		
2-methyl-1-butanol	57.0696	9.458	1226	1218	*	*	*
3-methyl-1-butanol	55.0542	9.499	1227	1218	*	*	*
ethyl hexanoate	88.0510	9.827	1236	1236	*	*	*
1-dodecene	69.0691	10.463	1253	1253			*
1-pentanol	55.0542	10.531	1255	1257	*	*	*
p-cymene	119.0860	10.886	1264	1265	*	*	*
methylpyrazine	94.0526	11.026	1268	1268		*	*
hexyl acetate	43.0192	11.301	1276	1276		*	*
cyclohexanone	55.0174	11.437	1279	1280		*	
2-octanone	58.0412	11.646	1285	1283	*	*	*
acetoin	45.0338	11.657	1285	1286		*	*
tridecane	57.0704	12.416	1305	1313	*	*	*
2,5-dimethylpyrazine	108.0679	12.899	1318	1316		*	*
2,6-dimethylpyrazine	108.0679	13.145	1325	1319		*	*
ethylpyrazine	107.0601	13.343	1330	1323	*	*	*
6-methyl-5-hepten-2-one	43.0184	13.663	1339	1339		*	
1-hexanol	56.0625	14.403	1359	1359	*	*	*
nonanal	57.0695	15.774	1395	1396	*		
tetradecane	57.0704	16.124	1405	1413		*	*
2-ethyl-3-methylpyrazine	121.0762	16.122	1405	1400		*	*
ethyl octanoate	88.0516	17.323	1438	1437	*	*	*
2,3-diethylpyrazine	136.0989	17.765	1451	1452	*	*	*
1-octen-3-ol	57.0336	18.017	1458	1456	*	*	*
acetic acid	60.0207	18.133	1461	1465		*	*
4-methyl-1-hexanol	70.0782	18.150	1461	1445		*	
furfural	96.0199	18.359	1467	1467	*	*	*
2,3-diethyl-5-methylpyrazine	150.1147	19.153	1489	1489	*	*	*
decanal	57.0693	19.464	1498	1498	*	*	*
2-ethyl-1-hexanol	57.0697	19.476	1498	1489	*	*	*
iodobenzene	77.0391	19.641	1503	1569	*	*	*
benzaldehyde	106.0410	20.148	1518	1521	*	*	*
(*E*)-2-nonenal	70.0421	20.651	1532	1546	*	*	*
furfuryl acetate	81.0341	21.013	1543	1541		*	
propanoic acid	43.047	21.078	1545	1545			*
linalool	71.0497	21.497	1557	1549	*	*	*
5-methyl-2-furaldehyde	110.0357	22.114	1575	1574	*	*	*
methyl decanoate	74.0368	22.912	1598	1598	*	*	*
hexadecane	71.0847	23.164	1605	1612	*		*
butyrolactone	86.0368	23.651	1619	1622			*
acetophenone	105.0340	24.263	1636	1636		*	*
butyric acid	60.0206	24.309	1637	1636		*	*
ethyl benzoate	105.0340	25.037	1658	1658	*	*	*
furfuryl alcohol	98.0360	25.285	1665	1666		*	*
geranyl formate	69.0704	25.437	1669	1665		*	*
2-methylbutyric acid	74.0357	25.757	1678	1674		*	*
3-methylbutyric acid	60.0205	25.764	1678	1679		*	*
alpha-terpineol	59.0497	26.356	1695	1688		*	*
heptadecane	71.0847	26.824	1706	1711	*		*
naphthalene	128.0626	27.598	1724	1723	*	*	*
geranyl acetate	69.0704	29.159	1759	1759		*	
citronellol	69.0704	29.777	1773	1774		*	*
benzothiophene	134.0190	30.319	1786	1799	*	*	*
geraniol	69.0704	31.112	1809	1800		*	
isopropyl dodecanoate	60.0201	32.696	1901	1841		*	*
hexanoic acid	60.0204	33.423	1944	1880	*	*	*
benzyl alcohol	79.0537	34.114	1984	1893	*	*	*
phenylethyl alcohol	91.0540	35.452	2041	1942	*	*	*
nonadecane	71.0848	35.676	2075	1991			*
octanoic acid	84.0197	38.099	2190	2100		*	
nonanoic acid	73.0296	38.660	2248	2171		*	
decanoic acid	73.0323	39.059	2271	2279		*	
Total compounds					34	61	54

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Table 2

LODs, LOQs and% RSDs of inter- and intraday repeatability parameters for the SAFE, SHS and SPME methodologies of volatile compounds usually found in cereal products.

volatile compound	SHS				SPME				SAFE			
	LOD (μg/kg)	LOQ (μg/kg)	% RSD Intraday	% RSD Interday	LOD (μg/kg)	LOQ (μg/kg)	% RSD Intraday	% RSD Interday	LOD (μg/kg)	LOQ (μg/kg)	% RSD Intraday	% RSD Interday
1-propanol	162	533	0.505	13.3	nd				nd			
hexanal	182	601	11.6	15.2	4.28	14.1	0.249	2.90	0.959	3.17	4.19	5.98
1-methylpyrrole	33.6	111	17.8	5.58	0.654	2.16	3.19	13.5	nd			
limonene	nd				0.131	0.432	4.29	6.15	1.57	5.17	3.32	0.512
heptanal	16.9	55.6	10.1	16.6	0.338	1.12	4.71	14.6	nd			
pyrazine	52.8	174	12.5	19.5	4.52	14.9	3.77	4.31	1.36	4.50	3.82	1.98
2-methyl-1-butanol	40.6	134	1.28	8.15	0.594	1.96	3.21	8.69	0.915	3.02	14.8	0.867
3-methyl-1-butanol	25.9	85.4	9.34	15.1	0.740	2.44	1.58	4.03	0.909	3.00	10.8	2.15
ethyl hexanoate	25.6	84.3	14.7	7.41	0.0840	0.277	6.90	5.16	0.569	1.88	1.66	4.68
1-pentanol	26.9	88.8	0.875	16.3	3.28	10.8	0.917	12.9	1.42	4.69	13.0	5.46
methylpyrazine	nd				0.133	0.439	1.44	9.02	0.473	1.56	2.73	6.70
hexyl acetate	nd				0.183	0.603	2.19	13.0	1.89	6.23	3.74	3.51
acetoin	nd				0.512	1.68	1.15	11.5	5.08	16.8	2.48	7.88
2-octanone	8.05	26.6	8.21	12.6	0.0812	0.268	5.77	14.8	0.341	1.13	2.74	3.60
2,5-dimethylpyrazine	nd				0.185	0.610	5.62	14.6	0.0954	0.315	1.89	1.38
2,6-dimethylpyrazine	nd				0.155	0.511	2.98	13.8	0.150	0.495	10.6	3.86
ethylpyrazine	nd				0.119	0.393	3.58	6.61	0.214	0.706	3.42	0.883
1-hexanol	17.4	57.3	7.21	12.1	2.07	6.84	3.30	8.03	0.214	0.706	3.97	0.932
2-ethyl-3-methylpyrazine					0.0465	0.153	2.76	1.32	0.120	0.397	2.06	1.07
nonanal	108	355	8.60	12.3	nd				nd			
ethyl octanoate	8.97	29.6	0.851	2.83	0.571	1.88	2.02	11.4	0.521	1.72	2.14	2.02
2,3-diethylpyrazine	6.93	22.9	9.88	15.4	0.0445	0.147	0.0123	10.5	0.135	0.446	0.208	2.07
1-octen-3-ol	7.02	23.2	19.2	10.9	0.0701	0.231	1.37	11.4	0.264	0.870	0.85	4.51
acetic acid	nd				8.01	26.4	6.73	11.4	0.347	1.15	0.367	5.43
furfural	36.9	122	19.4	10.9	0.360	1.19	1.79	9.20	0.440	1.45	0.00164	1.84
2,3-diethyl-5-methylpyrazine	3.32	11.0	11.3	15.2	0.0504	0.166	1.30	4.27	0.0930	0.307	1.22	3.43
2-ethyl-1-hexanol	21.3	70.4	1.78	9.59	2.59	8.54	1.55	2.97	0.533	1.76	2.47	2.43
benzaldehyde	30.5	101	7.60	11.0	8.35	27.6	2.10	11.1	0.278	0.916	0.257	2.80
(*E*)-2-nonenal	nd				0.130	0.429	1.67	4.05	1.11	3.65	2.66	4.20
furfuryl acetate	nd				0.211	0.695	2.09	13.1	nd			
5-methyl-2-furaldehyde	25.2	83.0	7.04	12.1	1.34	4.42	1.97	4.54	0.113	0.373	0.272	2.69
butyrolactone	nd				nd				0.727	2.40	3.87	4.53
butyric acid	nd				2.33	7.69	7.07	6.19	0.786	2.59	3.56	4.17
furfuryl alcohol	nd				2.74	9.04	2.87	3.99	0.316	1.04	2.29	4.86
2-methylbutyric acid	nd				1.17	3.86	2.62	2.46	0.485	1.60	0.129	3.49
3-methylbutyric acid	nd				1.17	3.87	4.73	5.97	0.505	1.66	1.75	3.03
hexanoic acid	nd				5.27	17.4	1.39	4.23	0.737	2.43	0.391	3.94
benzyl alcohol	773	2551	1.39	5.10	1.56	5.14	2.99	11.5	0.367	1.21	4.46	4.22
phenylethyl alcohol	nd				0.926	3.06	1.01	10.4	1.13	3.74	0.727	2.78
Average	76.8	253	8.62	11.8	1.53	5.04	2.86	8.43	0.725	2.48	3.35	3.37

nd = not detected.

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preter) and Kovats index. Additionally, the 39 volatile compounds in Table 2, all of which are included in Table 1, were also identified and confirmed by comparison of their retention times and accurate mass spectra with those of standards.

2.5. Study of the analytical performance of the proposed methods

The analytical parameters were evaluated following the AOAC guidelines (2002).

2.5.1. Limits of detection (LODs) and quantification (LOQs)

These parameters were calculated comparing the area of analyte peaks from a spiked corn starch sample and the area of the noise from a blank (solvent for SAFE or air for SHS and SPME) at the same retention time as that of the analyte peaks (all injected in triplicate).

2.5.2. Precision: Intraday repeatability and interday repeatability

For intraday repeatability, corn starch samples (without spiking) were injected in triplicate with the three methods and the RSD (%) of each compound was calculated. In terms of interday repeatability, corn starch samples were injected in triplicate on three alternate days and RSD (%) was calculated. Following the AOAC guidelines (2002), maximum RSDs of 8% were accepted for the repeatability.

2.5.3. Matrix effect and extraction efficiency

Both parameters were only calculated for the solvent extraction methodology, as they were impossible to calculate for the headspace methodologies because the equilibrium (solid-gas) of a spiked corn starch sample was not comparable with the equilibrium (liquid-gas) of the corresponding standard.

2.5.3.1. Matrix effect. The extract obtained from the SAFE distillation and concentrated below 1 mL with the Vigreux column was spiked and finally brought up to 1 mL. The signal of a sample without spiking (blank sample) was subtracted from the signal of the sample spiked after SAFE distillation and the resulting signal was compared with the corresponding standard mixture. Each injection was made in triplicate.

2.5.3.2. Extraction efficiency. This parameter was examined by comparing the recovery percentages with the matrix effect values. It is an indicator of the actual efficacy of the sample treatment. In order to estimate the recovery percentages, corn starch sample was spiked before the sample treatment (solvent extraction). The signal of a sample without spiking (blank sample) was subtracted from the signal of the sample spiked before the solvent extraction and the resulting signal was compared with the corresponding standard mixture. It was noted that extraction efficiencies should have been between 75% and 120% (AOAC guidelines, 2002).

2.5.4. Linearity and quantification of the corn starch sample by SPME-GC/QTOF

Because the SPME method was selected as the most suitable method for the analysis of the corn starch aroma, it was also employed in order to quantify and assess the linearity. For this purpose, a calibration curve was prepared using matrix-matched solutions in increasing concentrations within the range of 5–100 $\mu g\ kg^{-1}$. Six points were included in the calibration curves and a sample of corn starch without spiking was employed to subtract the blank signal. The coefficients of determination, r^2, were calculated.

2.6. Data analysis

The optimisation of the SHS and SPME methodologies was completed using central composite design (CCD) 3^3 with 3 central points (30 sets), Principal component analysis (PCA) and response surface method (RSM) (Ribeiro, Teófilo, Augusto, & Ferreira, 2010). CCD 3^3 with 3 central points and RSM were computed by the software Statgraphics Centurion version XVII (Statpoint Technologies, Warrenton, VA), while PCA was calculated using the software LatentiX version 2.00 (Latent5, Copenhagen, Denmark), with all the data auto-scaled prior to the analysis. In order to assess the variation of the volatile compounds between the different gluten-free flour bases, PCA was also conducted with the peak areas obtained by SPME-GC/QTOF, as the average of each flour/starch analysed in triplicate.

3. Results and discussion

3.1. Development and optimisation of the sample treatments

Corn starch was selected for development and optimisation since it was expected to present the lowest concentration of volatile compounds. Thus, if the methodology would detect the low amount of volatile compounds of corn starch, it would have enough sensitivity to be applied to more flavourful flours.

3.1.1. Solvent extraction methodology: SAFE

Initially, the Soxhlet extraction was conducted using 300 mL of a mixture of diethyl ether/dichloromethane (2:1) for 5 h at 40 °C (Pico, Nozal, Gómez, & Bernal, 2016). However, the recovery percentages were all below 40% (data not shown). Then, an 8-h extraction was tested and the signal of almost all the volatile compounds increased about three times (data not shown), with recovery percentages near 50%. However, a 15 h extraction led to losses of the very volatile compounds. Solvents with different polarities were also tested: acetone, dichloromethane, diethyl ether and hexane. The boiling point of hexane was too high and it was impossible to reduce the final volume to less than 2 mL, thus the sample was excessively diluted. The extraction with acetone led to extracts with solid residues of corn starch that leaked out from the Soxhlet thimble, probably due to the lixiviation of the starch in polar solvents. Moreover, when the acetone extract was reduced in the Vigreux column and cooled to room temperature, a gel was formed. This was probably due to the gelatinisation of the starch at room temperature (Ohwoavworhua & Osinowo, 2010). Regarding the use of dichloromethane, the recovery percentages slightly increased for some volatile compounds and decreased for others (data not shown). However, with diethyl ether the signal of almost all the volatile compounds was increased by about three times (data not shown). As a consequence, the most suitable extraction seemed to be with 300 mL of diethyl ether for 8 h. The SAFE conditions were previously selected employing a mixture of standards of the 35 volatile compounds, with recovery percentages near 100% for all the volatile compounds (data not shown). With these conditions, the volatile compounds that were detected are shown in Table 1. The extraction efficiencies of the 35 most common volatile compounds reported in the literature of cereal products are indicated in Table 3. In view of the possibilities regarding the solvents and the extraction time, these are the best results possible.

3.1.2. SHS

In order to optimise the weight of sample, the extraction temperature and the extraction time, a central composite design (CCD^3) with three central points and 30 sets was carried out, as is summarised in Table S1. Weight values higher than 3 g as well

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Table 3
Matrix effect (%) and extraction efficiency (%) of the SAFE methodology for 35 volatile compounds usually found in cereal products.

volatile compound	% matrix effect	% extraction efficiency
1-propanol	90.2	27.2
hexanal	103	58.8
limonene	91.8	24.7
pyrazine	87.6	24.2
2-methyl-1-butanol	85.3	36.0
3-methyl-1-butanol	88.7	39.2
ethyl hexanoate	102	28.4
1-pentanol	78.3	73.3
methylpyrazine	95.1	32.3
hexyl acetate	68.2	78.2
acetoin	103	88.8
2-octanone	80.3	77.1
2,5-dimethylpyrazine	101	47.3
2,6-dimethylpyrazine	104	53.5
ethylpyrazine	95.4	47.8
1-hexanol	74.6	84.9
2-ethyl-3-methylpyrazine	100	53.5
ethyl octanoate	94.1	64.1
2,3-diethylpyrazine	94.4	43.3
acetic acid	13.4	104
1-octen-3-ol	78.4	90.6
furfural	107	34.7
2,3-diethyl-5-methylpyrazine	103	65.7
2-ethyl-1-hexanol	65.7	81.2
benzaldehyde	104	44.2
(*E*)-2-nonenal	108	41.0
5-methyl-2-furaldehyde	45.0	102
butyrolactone	97.6	45.7
butyric acid	70.0	88.2
furfuryl alcohol	99.8	43.6
2-methylbutyric acid	103	59.5
3-methylbutyric acid	101	63.4
hexanoic acid	85.7	74.1
benzyl alcohol	95.8	72.8
phenylethyl alcohol	102	65.4
Average	89.1	58.8

as extraction times higher than 90 min did not significantly increase the peak area of the studied volatile compounds. Extraction temperatures higher than 120 °C were not tested, in order to avoid the *in situ* creation of volatile compounds by Maillard reactions (Onishi, Inoue, Araki, Iwabuchi, & Sagara, 2011). Firstly, PCA was employed in order to reduce the dimensionality of the original data (Ribeiro et al., 2010), since the maximum number of variables admitted in the typical statistical packages to carry out the "multiple responses optimisation" at one time is 16 (Rodriguez-Bencomo et al., 2012). Two principal components (PC1 and PC2) were sufficient to explain 82% of the variance of the original variables. The RSM was then applied to the values of the principal components of each volatile compound in the 30 experiments, as shown in Table S1. The optimum value of weight, extraction temperature and extraction time were 3 g, 120 °C and 71 min, respectively, as depicted in Fig. 1a. The "overall desirability function" (D), which estimates the suitability of the optimum for all the volatile compounds at once (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008), was 74.92%. Due to the high RSDs for the interday and intraday repeatability, which were higher than 10% and usually near 15%, 4-allylsyringol and 3,3-dimethyl-2-butanol were selected as internal standards. So as to avoid interferences between the solvent and the first volatile compounds of the chromatogram (very volatile solvent) or the last volatile compounds (low volatile solvent), dimethyl sulfide was selected. However, it was not possible to expose the spiked corn starch sample to the optimised conditions because the septum separated from the cap, due to the high volatility of the solvent. Thus, conditions with lower desirability function should be employed, if it is necessary to spike the sample, mainly with lower temperatures. With 3 g and 100 °C, the maximum desirability was achieved for 30 min (41% D function).

3.1.3. SPME

Firstly, four fibres were tested, including polydimethylsiloxane/divinylbenzene (PDMS/DVB) (65 μm), Carboxen/polydimethylsiloxane (CAR/PDMS) (85 μm), divinylbenzene/Carboxen/polydime thylsiloxane (DVB/CAR/PDMS) (50/30 μm) and polyacrylate (85 μm). All the fibres were cleaned at the temperature indicated by the manufacturer and blanks of air were run after each set of measurements, in order to ensure the absence of interferences. Due to its high polarity, polyacrylate was the fibre that detected the fewest number of volatile compounds and important compounds like 1-methylpyrrole, heptanal and hexyl acetate were not detected. Moreover, the peak areas of the remaining volatile compounds were the lowest of the four fibres (data not shown). The volatile compounds detected by DVB/CAR/PDMS and CAR/PDMS did not differ from one another, and only 2-octanone was lacking with PDMS/DVB (data not shown). CAR/PDMS yielded the highest peak areas, mainly for the volatile compounds of low and medium molecular weights, such as hexanal, 1-methylpyrrole, acetoin, acetic acid, benzaldehyde and 2-methylbutanoic acid. Meanwhile, PDMS/DVB primarily showed the highest peak areas for the volatile compounds of high molecular weight, such as 5-methyl-2-furaldehyde, benzyl alcohol or phenylethyl alcohol. DVB/CAR/PDMS presented the highest peak areas for 2-ethylpyrazine, 1-octen-3-ol and hexanoic acid and it yielded intermediate peak areas between PDMS/DVB and CAR/PDMS for the rest

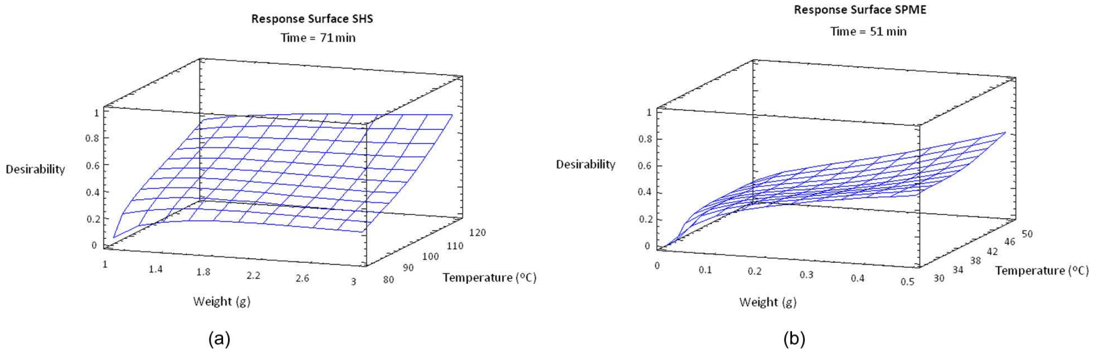

Fig. 1. Multiple response surfaces of the principal components (PCA) calculated from the peak areas arising from the central composite design (CCD) 3^3 with 3 central points. Response surface from PC1 and PC2 of the SHS methodology (Fig. 1a) and Response surface from PC1, PC2 and PC3 of the SPME methodology (Fig. 1b).

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of the volatile compounds. Therefore, DVB/CAR/PDMS was selected as the best option.

Similarly to SHS, a CCD^3 with three central points and 30 sets (Table S2), followed by PCA and RSM, was carried out for the optimisation of the weight, extraction temperature and extraction time, as summarised in Table S2. It was found that weight values higher than 0.5 g as well as extraction times higher than 120 min did not significantly increase the peak areas of the studied volatile compounds. Extraction temperatures higher than 50 °C have not usually been employed in SPME analyses of baked matrices and related products (Poinot et al., 2007; Ruiz, Quilez, & Guasch, 2003). In this case, three principal components (PC1, PC2 and PC3) were sufficient to explain 90% of the variance of the original variables, as shown in Table S2. The optimum values of weight, extraction temperature and extraction time were 0.5 g, 50 °C and 51 min, respectively, and the D function was 63%, as depicted in Fig. 1b.

3.2. Analytical characterisation of the developed methods

3.2.1. Number of detected volatile compounds

Sixty-one volatile compounds were identified with SPME, 54 with SAFE and only 34 with SHS (Table 1). SHS is limited to the analysis of abundant and very volatile compounds (Hui, Chen, & Nollet, 2010), justifying the lower number of detected volatile compounds in this study. On the other hand, a previous study by Mayuoni-kirshinbaum et al. (2012) indicated that SAFE extracts were richer in high-molecular weight volatile compounds, while SPME extracts were richer in low-molecular weight volatile compounds. Thus, 1-methylpyrrole, 2-heptanone and heptanal were found by SPME but not by SAFE, and hexadecane, heptadecane, nonadecane and butyrolactone were detected by SAFE but not by SPME. Surprisingly, octanoic, nonanoic and decanoic acids were found only by SPME. Pozo-Bayón, Guichard, and Cayot (2006) reported that acids are poorly extracted in cereal matrices, due to their strong interaction with amylose *via* hydrogen bonds, justifying the lack of detection of these acids in the SAFE extracts.

3.2.2. Facility of the sample treatment

It is well-known that headspace methodologies are solvent free, with easy preparation and sampling that is less time consuming, while solvent extraction methodologies are more tedious (Thompson-Witrick et al., 2015). The proposed SHS method requires 30 min of extraction while the proposed SPME method requires 51 min and scaling/weighing is the only manipulation of the sample in both cases. However, in order to use the SAFE methodology, around 11 h per sample is needed, along with a large volume of organic solvents. Moreover, the amount of corn starch required for SAFE is considerably larger than that required for headspace methodologies (50 g is needed for SAFE, 3 g for SHS and only 0.5 g for SPME).

3.2.3. LODs and LOQs

The average LODs of SPME and SAFE have reportedly been similar to one another (Majcher & Jeleń, 2009) and in the same range as our results (low μg kg^{-1}), explaining the high number of volatile compounds detected by both methodologies (Corral, Salvador, & Flores, 2015; Thompson-Witrick et al., 2015). The LODs and LOQs were much lower for SPME and SAFE than for SHS, ranging between 3.32 and 773 μg kg^{-1} for SHS, between 0.0445 and 8.35 μg kg^{-1} for SPME and between 0.0930 and 5.08 μg kg^{-1} for SAFE (Table 2). The higher LODs in the SHS methodology could be related to the lack of concentration of the volatile compounds once the equilibrium was reached.

3.2.4. Precision: Intraday repeatability and interday repeatability

The precision was calculated in order to evaluate if the results for the methods were similar on the same day (intraday repeatability) and over different days (interday repeatability). The RSDs for the intraday repeatability (Table 2) ranged from 0.505% to 19.4% for SHS (only nine lower than 8%), from 0.0123% to 7.07% for SPME and from 0.00164% to 14.8% for SAFE (only four higher than 5%). Thus, the use of internal standard (3,3-dimethyl-2-butanol/4-allyl syringol, 0.2 mg L^{-1} in dimethyl sulfide) was only mandatory for the SHS methodology. As previously explained, it is necessary to use conditions away from the optimum in order to calculate the precision parameters (3 g, 100 °C, 30 min).

The RSDs for the interday repeatability (Table 2) ranged from 2.83% to 13.1% for SHS, from 1.32% to 14.8% for SPME and from 0.512% to 7.88% for SAFE. Therefore, the use of internal standard (3,3-dimethyl-2-butanol/4-allylsyringol, 0.2 mg L^{-1}) was mandatory both for SHS and SPME.

In the end, the SAFE methodology was proven to be the most precise, probably because it implies injection of the same extract.

3.2.5. Extraction efficiency and matrix effect

These parameters were only calculated for the SAFE methodology, because there was no possibility to compare the solid-gas equilibrium of the sample headspace (SPME, SHS) with the liquid-gas equilibrium of the corresponding standard in solution; 300 mL of diethyl ether and 8 h of extraction were the most suitable conditions. However, 19 of the 35 volatile compounds presented recovery percentages lower than 50% (Table 3). Thus, as a potential explanation for this decreased signal, the possible impact of a matrix effect was studied. However, only for acetic acid, the low recovery percentage was due to a matrix suppression effect.

By adjusting the recovery percentages with the matrix effects, extraction efficiencies were obtained. Fifteen volatile compounds presented extraction efficiencies lower than 50%. The other 20 volatile compounds presented extraction efficiencies between 53.5% and 104%, with an average of 58.8%. Therefore, the SAFE methodology was not considered accurate enough for the analysis of volatile compounds in corn starch.

3.2.6. Selection of the most suitable method for the analysis of volatile compounds in corn starch

SHS presented important drawbacks due to the small number of detected volatile compounds, the inferior LODs and LOQs and the bad repeatability regarding SAFE and SPME. Therefore, SHS was excluded as a methodology to analyse volatile compounds in corn starch.

Regarding SPME and SAFE, the main difference between both methodologies was evident in the sample treatment. In summary, SPME involved an easy, quick (51 min) and solvent-free sample treatment that only required 0.5 g of corn starch. However, for the SAFE methodology, each sample required 11 h of experimentation, 300 mL of organic solvent, and 50 g of corn starch. Consequently, SPME was selected as the most suitable methodology for the analysis of volatile compounds in corn starch.

3.3. Quantification of volatile compounds in corn starch by SPME-GC/QTOF

The quantification was made with a calibration curve prepared using matrix-matched solutions (Table 4). In all cases, the coefficients of determination (r^2) were above 0.99.

A total of 35 volatile compounds was selected to be quantified (Table 4), compounds which have been reported as important contributors to the final aroma of cereal products, such as bread (Birch, Petersen, & Hansen, 2014; Pico, Bernal, & Gómez, 2015). Benzaldehyde, acetic acid, hexanoic acid and acetoin were found at concen-

Table 4
Parameters of the calibration curve in corn starch for the SPME methodology for 35 volatile compounds usually found in cereal products.

volatile compound	r^2	Added standard range (μg kg^{-1})	Concentration in sample (μg kg^{-1})	Origin[a]
hexanal	0.997	5.00–200	156 ± 0.389	L[b]
1-methylpyrrole	0.997	5.00–30.0	23.3 ± 0.743	nf[g]
limonene	0.994	5.00–100	82.0 ± 3.52	F[f]
heptanal	0.997	5.00–30.0	28.8 ± 1.36	L[b]
pyrazine	0.996	5.00–200	141 ± 5.30	L, M[d]
2-methyl-1-butanol	0.999	5.00–50.0	63.9 ± 2.05	F[b]
3-methyl-1-butanol	0.997	5.00–50.0	78.6 ± 1.24	F[b]
ethyl hexanoate	1.000	5.00–100	73.9 ± 5.10	F[b]
1-pentanol	0.990	5.00–300	282 ± 2.60	L[b]
methylpyrazine	0.997	5.00–50.0	21.7 ± 0.313	L, M[d]
hexyl acetate	0.999	1.00–50.0	2.37 ± 0.0519	F[b]
acetoin	0.996	1.00–50.0	1.44 ± 0.0166	F, M[b]
2-octanone	1.000	5.00–400	367 ± 21.2	L[c]
2,5-dimethylpyrazine	0.991	5.00–50.0	26.8 ± 1.51	L, M[d]
2,6-dimethylpyrazine	0.998	5.00–50.0	20.9 ± 0.622	L, M[d]
ethylpyrazine	1.000	5.00–50.0	16.8 ± 0.600	L, M[d]
1-hexanol	0.993	5.00–100	86.6 ± 2.86	L[b]
2-ethyl-3-methylpyrazine	0.999	5.00–30.0	9.56 ± 0.264	L, M[d]
ethyl octanoate	0.995	5.00–50.0	53.2 ± 1.07	F[b]
2,3-diethylpyrazine	0.994	5.00–50.0	16.5 ± 0.00165	L, M[d]
1-octen-3-ol	0.992	5.00–50.0	99.6 ± 1.36	L[b]
acetic acid	0.996	5.00–100	17.4 ± 1.17	F, M[b]
furfural	0.992	5.00–30.0	6.27 ± 0.112	F, M[b], L[e]
2,3-diethyl-5-methylpyrazine	0.999	5.00–50.0	24.0 ± 0.31	L, M[d]
2-ethyl-1-hexanol	0.994	5.00–200	105 ± 1.62	L[h]
benzaldehyde	0.996	5.00–50.0	17.9 ± 0.377	F, M[b], L[b,h]
(*E*)-2-nonenal	0.991	5.00–50.0	28.6 ± 0.478	L[b]
5-methyl-2-furaldehyde	0.997	5.00–50.0	80.3 ± 1.58	F[b]
butyric acid	1.000	5.00–30.0	72.1 ± 5.10	F[b,i], L[i]
furfuryl alcohol	0.997	10.0–100	28.6 ± 0.819	L[h]
2-methylbutyric acid	0.995	5.00–30.0	29.2 ± 0.765	F[b,i], M, L[i]
3-methylbutyric acid	1.000	5.00–30.0	10.2 ± 0.484	F[b,i], M, L[i]
hexanoic acid	0.993	5.00–30.0	17.2 ± 0.239	F, L[b]
benzyl alcohol	0.995	5.00–100	16.4 ± 0.490	L, M[b]
phenylethyl alcohol	0.998	5.00–50.0	10.5 ± 0.106	F[b]

[a] L = lipid oxidation; F = fermentation; M = Maillard.
[b] Pico et al. (2015).
[c] Bett and Boylston (1992).
[d] Eskin and Shahidi (2012).
[e] Frankel (2005).
[f] Jongedijk et al. (2016).
[g] nf = not found.
[h] Sayaslan (2005).
[i] Birch et al. (2014).

trations below their LOQs. Conversely, 2-octanone, 1-pentanol, hexanal, pyrazine and 2-ethyl-1-hexanol were found in higher concentrations, being 367 μg kg^{-1}, 282 μg kg^{-1}, 156 μg kg^{-1}, 141 μg kg^{-1} and 105 μg kg^{-1}, respectively. All of them are products from lipid oxidation (Bett & Boylston, 1992; Pico et al., 2015; Sayaslan, 2005). The other 26 volatile compounds, ranging between 1.44 μg kg^{-1} and 99.6 μg kg^{-1}, were considered ultra-trace. This is in concordance with the work reported by Sayaslan (2005), who explained that most of the cereal starch odours have been attributed to the autoxidation of lipids present on the surface of the starch granules. Indeed, 20 of the 35 quantified volatile compounds could have been generated by lipid oxidation (Table 4). Pyrazines result from the reaction between Maillard reaction products and lipid oxidation products (Eskin & Shahidi, 2012). This reaction could have taken place during the processing of corn grain, since the germ of the grain contains a sufficient amount of lipids and there is heat in the medium to carry out Maillard reactions. Moreover, some Maillard products can be generated during the long storage times typical of cereal grains, since the Maillard reaction takes place at room temperatures with low kinetics (Benzing-Purdie, Ripmeester, & Ratcliffe, 1985).

Hexanal has been reported as the most abundant compound in corn, potato and wheat starches (Sayaslan, 2005). However, hexanal was the third most abundant in our corn starch sample, preceded by 1-pentanol and the most abundant 2-octanone. Nevertheless, 2-octanone was not reported in Sayaslan's work, which could be attributed to the differences in extraction technique employed (they used DHE). 2-Octanone has only been reported in pea flour (Murat et al., 2012), although it was found only by SAFE and purge-and-trap. Additionally, heptanal, benzaldehyde and (*E*)-2-nonenal were also reported in Sayaslan (2005). Finally, 2,3-butanedione, hexanal, acetic acid, butyric acid, 2-methylbutyric acid and 3-methylbutyric acid have also been reported in wheat flour (Czerny & Schieberle, 2002) and rye flour (Kirchhoff & Schieberle, 2002).

3.4. Qualitative comparison of different gluten-free flour basis by SPME-GC/QTOF

Seventy-three volatile compounds were found among the four flours (quinoa, buckwheat, teff and rice) and corn starch (Table S3), although only 16 of them were common among the examined samples. Hexanal, 3-methyl-1-butanol, 1-pentanol, 1-octen-3-ol, acetic acid, furfural, benzaldehyde, (*E*)-2-nonenal and phenylethyl alcohol were common to all the flours and starch

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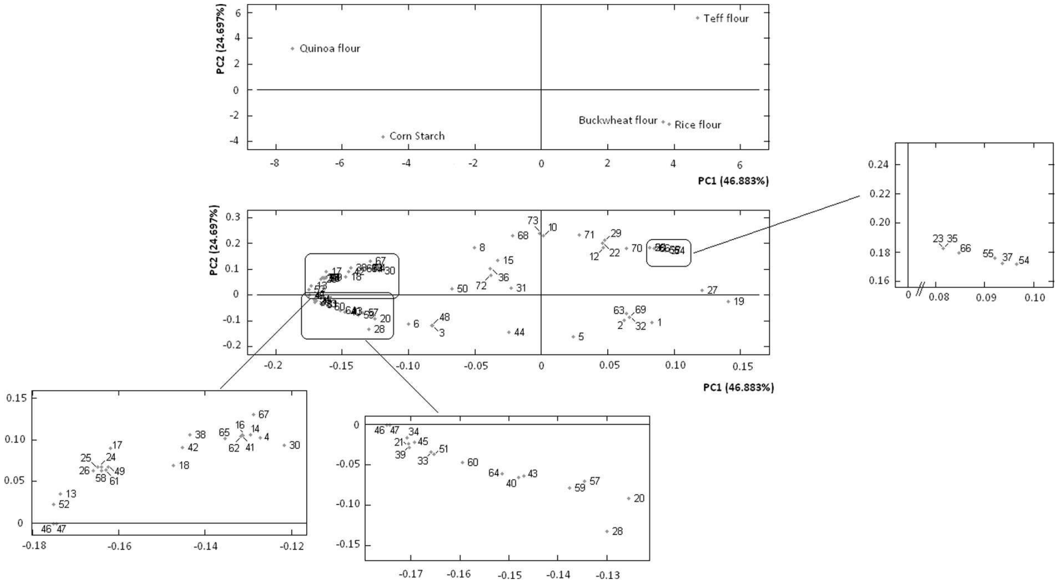

Fig. 2. PCA of the gluten-free flours and corn starch analysed by SPME-GC/QTOF. The scores plot represents the five samples and the loadings plot represents the 73 volatile compounds found across all the flours and starches. The numbers corresponding to each volatile compound are indicated in Table S3.

and have usually been reported in the literature for cereal products (Birch et al., 2014; Pico et al., 2015).

In order to better understand the difference between the flour bases, a PCA of the 73 volatile compounds was calculated (Fig. 2). Regarding the scores plot, there is a clear separation between quinoa flour and corn starch (negative component of the PC1) and teff, buckwheat and rice flours (positive component of the PC1). Only buckwheat and rice were located in the same area, which means that they presented similar volatile profiles. Taking PC1 into consideration, which explains 46.8% of the variability of the original values, quinoa flour and corn starch presented the highest abundances in 53 of the 73 volatile compounds. Regarding the loadings plot, quinoa flour was distinguished by the highest contents in methyl, ethyl and dimethyl pyrazines, terpenes, benzyl alcohol, phenylethyl alcohol, ethyl hexanoate and hexyl acetate. Corn starch presented the highest content in diethyl pyrazines, alcoholic terpenes, benzaldehyde, furfural and ethyl octanoate, furfuryl acetate, methyl decanoate, geranyl formate and geranyl acetate. Meanwhile, buckwheat was characterised by the highest content in acetoin and rice in 4-vinylguaiacol. Finally, teff was characterised by the highest contents of 3-/2-methyl-1-butanol, 1,3-butanediol, as well as acetic acid, 3/2-methylbutanoic acid and hexanoic, octanoic and nonanoic acids. Nevertheless, none of the flours/starch was characterised by a high number of volatile compounds from lipid oxidation: (i) quinoa flour presented the highest contents of 1-pentanol, 1-octen-3-ol and 2-ethyl-1-hexanol; (ii) corn starch was highest in heptanal and (*E*)-2-nonenal; (iii) teff flour had the highest levels of 1-hexanol and (*Z*)-2-heptenal; (iv) rice flour was highest in (*Z*)-2-octenal, pentanal and hexanal.

4. Conclusions

Three different analytical methodologies were evaluated for the analysis of volatile compounds in corn starch. SHS-GC/QTOF was excluded due to the low number of volatile compounds detected. Regarding SPME and SAFE, both presented suitable sensitivity and resulted in detection of a similar number of volatile compounds. However, SAFE required 300 mL of organic solvent, 50 g of sample and 11 h of analysis, while SPME was solvent-free and required 0.5 g of sample and 51 min of extraction. Therefore, SPME-GC/QTOF was selected as the most suitable methodology.

The SPME-GC/QTOF method was successfully utilised for the quantification of 35 selected volatile compounds in corn starch, with 20 of them resulting from lipid oxidation. Finally, SPME-GC/QTOF was also applied for making a comparison of the volatile profiles of gluten-free flour bases. Quinoa flour and corn starch were characterised by the highest contents of pyrazines, terpenes, benzenic compounds and esters, while teff, buckwheat and rice flours were distinguished by the highest contents of 3/2-methyl-1-butanol, acetoin, 4-vinylguaiacol and organic acids.

The proposed SPME-GC/QTOF methodology could potentially be applied to the quality control of stored flours, in order to avoid the appearance of off-flavours. It also could be used to detect faults in the process of flour production, due to the appearance of new volatile compounds that are not present in the usual volatile profile.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.06.157.

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Analysis of volatile compounds in gluten-free bread crusts with an optimised and validated SPME-GC/QTOF methodology

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ABSTRACT

The aroma of bread crust, as one of the first characteristics perceived, is essential for bread acceptance. However, gluten-free bread crusts exhibit weak aroma. A SPME-GC/QTOF methodology was optimised with PCA and RSM and validated for the quantification of 44 volatile compounds in bread crust, extracting 0.75 g of crust at 60 °C for 51 min. LODs ranged between 3.60 and 1760 $\mu g\,Kg^{-1}$, all the R^2 were higher than 0.99 and %RSD for precision and %Er for accuracy were lower than 9% and 12%, respectively. A commercial wheat bread crust was quantified, and furfural was the most abundant compound. Bread crusts of wheat starch and of japonica rice, basmati rice and teff flours were also quantified. Teff flour and wheat starch crusts were very suitable for improving gluten-free bread crust aroma, due to their similar content in 2-acetyl-1-pyrroline and 4-hydroxy-2,5-dimethyl-3(2H)-furanone compared to wheat flour crust and also for their high content in pyrazines.

1. Introduction

The aroma of bread crust is one of the first attributes sensed when entering a bakery shop. It has been characterised by volatile compounds from Maillard reactions, caramelisation and thermal degradation (Pico, Bernal, & Gómez, 2015), although there can be volatile compounds from lipid oxidation in smaller proportions (Moskowitz, Bin, Elias, & Peterson, 2012). 2-Acetyl-1-pyrroline, generated by Maillard reactions, has been considered the key volatile compound of wheat flour bread crust. Other important volatile compounds include 3-methylbutanal, 2,3-butanedione and 4-hydroxy-2,5-dimethyl-3(2H)-furanone, also from Maillard reactions, along with 2-(E)-nonenal and 2,4-(E,E)-decadienal from lipid oxidation (Zehentbauer & Grosch, 1998).

In the case of gluten-free bread, the sensory quality is barely acceptable, almost notably the texture and the aroma (Pacyński, Wojtasiak, & Mildner-Szkudlarz, 2015). Quality parameters such as nutritional value, rheology of the dough, texture, volume and colour have been widely studied in gluten-free bread (Houben, Höchstötter, & Becker, 2012; Masure, Fierens, & Delcour, 2016). However, there is little knowledge regarding the aroma of gluten-free bread crusts. To our knowledge, only Pacyński et al. (2015) have studied the volatile compounds of gluten-free bread crusts with amino acid – sugar pairs added with the aim of promoting the generation of Maillard compounds and improving the aroma of the crust.

Therefore, the analysis of volatile compounds of bread crust becomes essential in order to improve bread quality, above all of gluten-free bread crusts. In the last decade, solid phase microextraction (SPME) combined with GC/MS has been preferred because it is a quick, simple and solvent-free technique (Thompson-Witrick et al., 2015). Moreover, it only requires a minimal amount of sample, which is important in the case of gluten-free breads that present a poor crust. Focusing on SPME-GC/MS volatile compounds analyses, most researchers have studied the crumb and crust together (Paraskevopoulou, Chrysanthou, & Koutidou, 2012; Plessas et al., 2008, 2011; Poinot et al., 2007, 2008). The study of the volatile compounds from the crust separately from the crumb is very important in order to understand its volatile profile. To our knowledge, only Raffo, Carcea, Castagna, and Magrì (2015) and Pacyński et al. (2015) have studied the volatile compounds of bread crust by SPME-GC/MS, the latter examining gluten-free bread crust. On the other hand, understanding the performance characteristics of the analytical methodology is crucial in order to achieve reliable results, but this information has only been reported for SPME-GC/MS analyses of bread by Raffo et al. (2015). They studied the repeatability, intermediate precision, linearity as well as LOD and

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Abbreviations: 2-ACPY, 2-acetyl-1-pyrroline; CAR, carboxen; CCD, central composite design; D, desirability function; DVB, divinylbenzene; DOE, design of experiments; FD, flavour dilution factor; GC/QTOF, gas chromatography/quadrupole-time of flight; HPMC, hydroxyl propyl methyl cellulose; LOD, limit of detection; LOQ, limit of quantification; MSA, method of standard addition; OT, odour threshold; PA, polyacrylate; PC, principal component; PCA, principal component analysis; PDMS, polydimethylsiloxane; R^2, coefficient of determination; Re, relative error; RSD, relative standard deviation; RSM, response surface method; SPME, solid-phase microextraction

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LOQ for volatile compounds analyses in wheat bread crust. However, to the best of our knowledge, the accuracy has not been studied for any SPME-GC/MS methodology; verifying the accuracy is very important for interpreting the quantifications made from these methodologies, since it expresses the closeness of the experimental result to the accepted value (AOAC guideline, 2002). Finally, the optimisation of the methodology before its validation is also imperative so as to ensure that the maximum amount of analyte is extracted, but any optimisation was carried out by Raffo et al. (2015) for the analysis of the volatile compounds of the crust by SPME-GC/QTOF. Moreover, as far as we know, the use of statistical tools such as the Response Surface Method (RSM) has not been reported for the optimisation of SPME methodologies for bread volatile compounds analyses.

Therefore, the first aim of this study was to optimise and validate a SPME-GC/quadrupole-time-of-flight (QTOF) methodology for the semi-quantification (lower limits of detection, since it works in splitless mode) and quantification (higher limits of detection, since it works in split mode) of 44 volatile compounds in bread crust, employing a commercial bread crust sample for this purpose. The quantification of the commercial sample was made using the Method of Standard Addition (MSA). It must be noted that this is the first time that a SPME methodology has been optimised through the use of Design of Experiments (DOE) in the analysis of volatile compounds in bread, specifically with Principal Component Analysis (PCA) followed by RSM. The second goal was to quantify volatile compounds through the MSA of teff, basmati rice, japonica rice and wheat starch bread crusts for the selection of the most suitable gluten-free flour or starch for the improvement of the final aroma of gluten-free bread crust, using wheat bread as a control sample. The choice of the quantified gluten-free bread crusts was made using the semi-quantification method as screening process of oat, quinoa, teff, basmati rice, japonica rice and corn and wheat starch.

2. Materials and methods

2.1. Materials, reagents and standards

For the analytical characterisation of the method, 2-acetyl-1-pyrroline (2-ACPY) was purchased from Eptes (Vevey, Switzerland) and the other 43 pure standards found in Table 1 were purchased from Sigma-Aldrich (Steinheim, Germany). Dichloromethane was obtained from Scharlab (Barcelona, Spain) and methanol was from VWR International (Fontenay-sous-Bois, France). Argon, nitrogen and helium were acquired from Carburos Metálicos (Barcelona, Spain).

2.2. Preparation of standard solutions

2-ACPY solutions were prepared in dichloromethane, as 2-ACPY dimerises in methanol and water. It was necessary to work under inert atmosphere of argon at all times due to the compound's lack of stability to oxygen and moisture. For this reason, dichloromethane was dried in a SDS PS-MD-5 purification system from Düperthal Sicherheitstechnik (Karlstein am Main, Germany). For the other 43 volatile compounds included in Table 1, working solutions of each volatile compound were prepared in methanol. All the solutions were stored in a freezer at −20 °C.

2.3. Sample employed for the development of the SPME-GC/QTOF method

The development and characterisation of the methodology were carried out with the crust of wheat bread purchased from Forvasa (Puçol, Spain). The label indicated that the ingredients were wheat flour, water, salt, yeast and flour improver (wheat flour, anti-caking agent (E-170), emulsifier (E-472e), antioxidant (E-300) and enzymes).

Loaves of bread were cut into slices of 5 cm width, including the ends. The crust was scratched with a knife, taking care not to remove pieces of crumb. Once all the crust was removed, it was frozen with liquid nitrogen and finally it was grounded in an Ika grinder model M20 (Staufen, Germany) for 10 s.

Table 1
LODs and LOQs (in μg Kg^{-1}), repeatability (%RSD) and accuracy (%Re) of the 44 studied volatile compounds with proposed quantitative method. The LODs of the semi-quantitative method are also given.

Volatile compound	LOD split 1:100	LOQ split 1:100	LOD splitless	% RSD intraday	% RSD interday	% Re
2,3-Butanedione	131	438	0.801	2.24	4.60	3.33
Hexanal	62.8	209	2.53	2.39	4.40	1.82
2-Methyl-1-propanol	57.6	192	1.38	0.480	4.60	3.59
1-Methylpyrrol	28.6	95.2	1.49	0.400	0.800	3.15
Heptanal	101	338	1.27	1.65	1.50	3.18
R-Limonene	5.70	19.0	2.54	0.780	6.50	4.14
Pyrazine	54.2	181	3.42	0.750	5.60	1.47
2-Methyl-1-butanol	128	427	0.77	5.75	4.80	2.12
3-Methyl-1-butanol	140	467	0.78	3.96	5.10	0.977
1-Pentanol	402	1340	1.00	6.23	9.00	1.57
2-Methylpyrazine	53.6	179	0.15	0.530	1.30	0.324
Acetoin	476	1586	1.49	2.73	2.90	0.821
2-Octanone	40.2	134	0.34	4.52	4.70	10.9
2,5-Dimethylpyrazine	22.7	75.5	0.18	4.95	3.70	3.27
2,6-Dimethylpirazine	35.0	117	1.39	3.73	4.90	1.93
2-Ethylpyrazine	28.6	95.5	0.24	0.670	2.30	0.847
2-Acetyl-1-pyrroline	12.1	39.9	0.19	1.25	3.21	0.981
2,3-Dimethylpyrazine	25.6	85.3	2.53	0.500	4.10	2.57
1-Hexanol	105	349	0.81	0.760	2.10	1.55
Nonanal	102	341	2.47	2.57	3.70	0.235
2,3,5-trimethylpyrazine	4.70	15.6	1.76	0.570	4.50	1.31
2-Ethyl-3-methylpirazine	8.00	26.6	1.90	0.930	4.30	2.34
Ethyl octanoate	3.60	12.0	0.65	5.39	8.60	2.55
1-Octen-3-ol	8.40	27.9	3.67	3.58	3.50	0.281
Acetic acid	49.3	164	1.62	7.03	1.00	nq[a]
Furfural	50.3	168	3.61	2.38	2.70	7.02
2-Ethyl-1-hexanol	22.6	75.4	2.01	5.17	4.80	0.648
Benzaldehyde	19.7	65.7	1.84	0.830	4.10	0.085
2-(E)-Nonenal	43.6	146	1.91	1.13	3.10	12.2
5-Methyl-2-furaldehyde	87.7	292	0.21	0.130	2.40	3.94
Butyrolactone	743	2477	1.38	4.42	7.40	5.14
2-Acetilpyrazine	15.3	51.0	0.95	1.27	4.90	2.56
Butyric acid	392	1307	0.81	1.31	2.50	1.58
Phenylacetaldehyde	28.50	94.8	0.68	1.32	2.20	0.683
Furfuryl alcohol	66.6	222	1.93	1.84	4.10	nq[a]
2-Methylbutyric acid	225	751	4.46	5.51	1.20	2.58
3-Methylbutyric acid	667	2224	3.17	7.89	1.50	0.229
2,4-(E,E)-Decadienal	25.9	86.3	1.73	5.20	4.30	3.05
Hexanoic acid	1540	5132	0.85	0.120	2.40	0.374
Benzyl alcohol	67.8	226	0.77	0.560	7.20	1.66
Phenylethyl alcohol	48.8	163	2.76	3.91	8.60	0.425
2-Acetylpyrrol	290	966	1.34	3.86	4.90	1.68
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	1755	5851	3.61	3.74	2.70	0.945
4-Vinylguaiacol	985	3284	3.15	3.73	5.20	1.83

[a] nq = not quantified.

2.4. Gluten-free bread formulation: flours, starches, hydrocolloid and yeast

Wheat starch was supplied by Roquette Laisa (Valencia, Spain), corn starch by Miwon Daesang (Seul, Korea) and wheat flour by Harinera Castellana (Medina del Campo, España). Japanica rice flour was purchased from Molendum ingredients (Zamora, Spain), oat flour from Emilio Esteban (Valladolid, Spain), quinoa flour from El Granero Integral (Madrid, Spain) and teff flour from Salutef (Palencia, Spain). Basmati flour was milled from basmati rice from Dacsa (Lisboa, Portugal), employing a grinder model Perten 3300 (Hägersten, Sweden). Hydroxyl propyl methyl cellulose (HPMC) K4M was supplied by Dow Chemicals (Michigan, USA) and the dry baker's yeast (*Saccharomyces cerevisiae*) by Lesaffre (Cerences, France). All yeasts belonged to the same batch to decrease the risk of different cell count of yeast and different contaminant bacteria.

2.5. Gluten-free bread making

The following ingredients, as g/100 g of flour or starch, were used in all the formulas: sunflower oil (6 g/100 g), sucrose (5 g/100 g), salt (1.8 g/100 g), yeast (3 g/100 g), HPMC (2 g/100 g) and water (100 g/100 g). They were mixed using a Kitchen-Aid Professional mixer (KPM5, KitchenAid, St. Joseph, Michigan, USA) for 8 min at speed of 56 rpm. The fermentation was carried out for 90 min in a chamber at 30 °C with 90% of relative humidity, reaching average specific volumes between 1.91 mL/g and 6.89 mL/g (data not shown). The doughs were baked, in rows of two, at 190 °C for 40 min in a convection oven model Salva 5 grid (Guipuzcoa, Spain). After baking, the gluten-free breads were left at room temperature for 30 min and cut as described in Subsection 2.3. Each sample was prepared in duplicate (n = 2).

2.6. Solid-phase microextraction

Four fibres were tested, including polydimethysiloxane/divinylbenzene (PDMS/DVB) (65 μm), carboxen/polydimethysiloxane (CAR/PDMS) (85 μm), divinylbenzene/carboxen/polydimethysiloxane (DVB/CAR/PDMS) (50/30 μm) and polyacrylate (85 μm), all of them from Sigma Aldrich (Gillingham, UK). The selected fibre was DVB/CAR/PDMS and an autosampler was employed for the extraction of the volatile compounds. An amount of 0.75 g (± 0.0050 g) of wheat bread crust was weighed into a 20 mL vial and sealed with a magnetic screw cap provided with PTFE/silicone septa. The sample was incubated in the oven for 5 min at 60 °C (without the fibre) and then the volatile compounds were extracted in the same oven for 51 min at 60 °C, without agitation. After that, the fibre was inserted into the GC injector port for thermal desorption for 5 min at 270 °C, with an injection volume of 1 μL. Finally, the fibre was conditioned for 30 min at 270 °C after each analysis.

2.7. GC/QTOF chromatographic conditions

GC/QTOF analyses were performed on a 7890A gas chromatograph coupled to a 7200 Quadrupole-Time of flight (QTOF) mass spectrometer detector and MassHunter B.07.00 software, all from Agilent Technologies (Santa Clara, California, USA). The GC was equipped with a CombiPAL RSI 85 autosampler from CTC Analytics AG (Zwingen, Switzerland). The separation was achieved on a polar Innowax column (100% polyethylene glycol, 30 m × 0.25 mm ID × 0.25 μm) obtained from J&W Scientific (Agilent Technologies, California, USA). The chromatographic conditions were previously optimised by the research group using standard solutions (Pico, del Nozal, Bernal, & Gómez, 2017). The GC was operated under programmed temperature conditions: from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, then the temperature was increased to 114 °C (6.7 min) at 1 °C/min, afterwards it was increased to 136 °C (0 min) at 2.5 °C/min and finally it was increased to 245 °C (5 min) at 85 °C/min. Total run time was 43 min. The carrier gas was helium at a flow rate of 1.1 mL/min. The injector temperature was 270 °C, working in splitless mode for semi-quantitative analyses and in split mode for quantitative analyses. When the sample was spiked in the quantitative analysis using MSA, the most abundant compounds saturated the detector, thus it was compulsory to dilute the sample working in split mode. If the sample was spiked with less concentration, the increase in the signal was not sufficient to achieve good quantification. However, when the sample was not spiked there was no saturation and it was possible to work in splitless mode, increasing the sensitivity. The use of two working modes for different compounds was possible because the same volatile compound was studied in all the samples, which were injected in the same mode. However, different compounds injected in different modes were not compared. The interface, ion source and quadrupole temperatures were 250 °C, 230 °C and 150 °C, respectively. Analyses were performed in SCAN mode and included a mass range of 20–350 *m/z*, operating in electron ionization mode with energy of 70 eV. All the 44 volatile compounds shown in Table S1 were identified by comparison of their retention times and accurate mass spectra (with four decimal places) with standards as well as using their Kovats Index (Table S1) and their Mass Spectra Library (NIST MS Search 2.2 & MS Interpreter).

2.8. Validation of the SPME-GC/QTOF method

The analytical parameters were evaluated following the AOAC guidelines (2002).

2.8.1. Limits of detection (LODs) and quantification (LOQs)

These parameters were calculated comparing the area of analyte peaks from a spiked crust sample and the area of the noise from a blank (the air of an empty vial) at the same retention time as that of the analyte peaks. Injections were made in quintuplicate (n = 5). LODs were calculated as 3 times the signal to noise ratio (S/N), while LOQs were calculated as 10 times the S/N.

2.8.2. Precision: intra-day repeatability and inter-day repeatability

For intra-day repeatability, crust samples were injected in quintuplicate and the RSD (%) of each compound was calculated (n = 5). In terms of inter-day repeatability, crust samples were injected in quintuplicate on three alternate days and RSD (%) was calculated (n = 5). Following the AOAC guidelines (2002), maximum RSDs of 15% were accepted for the repeatability.

2.8.3. Quantification of volatile compounds of the commercial wheat bread crust sample: linearity and accuracy

The quantification was made using the MSA. A matrix-matched calibration curve was made spiking six aliquots of the commercial crust sample with increasing concentration of the standard mixture (which contains the 44 volatile compounds) within the range of 0.150–1.30 mg Kg^{-1}. Six points were included in the calibration curve. The coefficients of determination R^2 were obtained and a *t*-test for the linearity was done in order to ensure the linear tendency of the regression ($t_{experimental} > t_{critical}$). The relative errors (% Re) for the accuracy were also calculated. Following the AOAC guidelines (2002), maximum Re of 15% were accepted for accuracy. The % Re, for each volatile compound, was examined using the theoretical concentration calculated with the regression curve from an intermediate signal and the experimental concentration that gives this intermediate signal. % Re was given as: $\frac{Ct - Ce}{Ce} \times 100$, where Ct means “theoretical concentration” and Ce “experimental concentration”.

2.9. Application of the SPME-GC/QTOF method to the quantification of the volatile compounds of gluten-free bread crusts

The quantification was made in the same way than for the commercial bread, using the MSA. The volatile compounds of the crusts made with basmati rice, japonica rice and teff flours and wheat starch, as well as wheat flour (control sample), matrix-matched calibration curves were made in the range of 0.150–9.00 mg Kg^{-1} for all the volatile compounds except for 2-ACPY, which was from 0.0200–0.960 mg Kg^{-1}. The same procedure as that in Sub-sections 2.6–2.7 was followed.

2.10. Statistical analysis of the data

The optimisation of the SPME methodology was made using a modification of a Central Composite Design (CCD) 3^3 (9 experiences), PCA and RSM. The modification of the CCD 3^3 as well as the RSM were computed by the software Statgraphics Centurion version XVII (Statpoint Technologies, Warrenton, Virginia), while PCA was done with the software LatentiX version 2.00 (Latent5, Copenhagen, Denmark), with data standardized prior to the analysis. In order to assess the variation of the volatile compounds among the different gluten-free bread crusts both in the semi-quantitative and quantitative studies, PCA were also conducted, which were the average of each bread crust sample prepared in duplicate and analysed in triplicate (n = 6). In the case of the semi-quantitative studies, the peak areas were employed to perform the PCA; while in the case of the quantitative studies, the concentrations in $\mu g\,Kg^{-1}$ were the data employed for the PCA.

3. Results and discussion

3.1. Optimisation of the SPME conditions

3.1.1. Selection of the fibre

Four fibres were tested, including PDMS/DVB, CAR/PDMS, DVB/CAR/PDMS and polyacrylate. The conditions employed for the selection of the fibre were that of the set 1 of Table S2. All the fibres were cleaned at the temperature indicated by the manufacturer and blanks of air were run after each set of measurements in order to ensure the absence of interferences. Due to its high polarity, polyacrylate was the fibre that detected the fewest number of volatile compounds (Table S1) and important compounds like 2,3-butanedione, 1-methylpyrrol, heptanal or 2,3-dimethylpyrazine were not detected. Moreover, the peak areas of the remaining volatile compounds were the lowest of the four fibres. For PDMS/DVB, only 2-octanone was lacking and CAR/PDMS as well as DVB/CAR/PDMS detected the 44 volatile compounds. CAR/PDMS yielded the highest peak areas for the volatile compounds of low and medium molecular weights, such as hexanal, 1-methylpyrrol, acetoin, acetic acid, benzaldehyde or 2-methylbutanoic acid. Meanwhile, PDMS/DVB primarily showed the highest peak areas for the volatile compounds of high molecular weight, such as benzaldehyde, 2-(E)-nonenal, phenylacetaldehyde, benzyl alcohol or phenylethyl alcohol. DVB/CAR/PDMS presented the highest peak areas for 2,3-butanedione, 2-ethylpyrazine, 2-acetylpyrazine, 1-octen-3-ol and hexanoic acid and it yielded intermediate peak areas between PDMS/DVB and CAR/PDMS for the rest of the volatile compounds. Therefore, DVB/CAR/PDMS was selected as the best option for detection of volatiles compounds in gluten-free bread crusts.

3.1.2. Selection of the weight, extraction time and extraction temperature

In order to optimise the weight of sample, the extraction temperature and the extraction time, a modification of the Central Composite Design (CCD^3) was carried out (Table S2). The starting point of the design was the optimum (set 1, Table S2) obtained from a Central Composite Design (CCD^3) with three central points for the optimisation of a SPME-GC/QTOF methodology for the analysis of volatile compounds in flours and starches (Pico, Tapia, Bernal, & Gómez, 2017), since the moisture content of the flours and starches was not expected to be much higher than that of the crust (the moisture of our bread crusts ranged between 7.07% and 11.74%, data not shown). Nine combinations or sets of values of weight, extraction time and extraction temperature higher and lower than the optimum were used for experimentation (Table S2). The maximum value of weight (0.75 g) was selected in order to avoid the saturation of the detector, and the minimum (0.25 g) was selected due to the great losses of signal with lower weights. The maximum value of temperature (70 °C) was chosen so as to avoid the development of Maillard reactions, but with lower temperatures (50 °C) the signal decreased a lot. Finally, the minimum value of time (30 min) was selected due to the great losses of signal at lower times and the maximum time (75 min) because at higher times there was not visible increase of the signal.

PCA was employed in order to reduce the dimensionality of the original data (Ribeiro, Teófilo, Augusto, & Ferreira, 2010). Two principal components (PC1 and PC2) were sufficient to explain 86.2% of the variance of the original variables. The RSM was then applied to the values of the two principal components of each volatile compound in the 9 experiments, as shown in Table S2. The optimum values of weight, extraction temperature and extraction time were 0.750 g, 60 °C and 51 min, respectively. The "overall desirability function" (D), which estimates the suitability of the optimum for all the volatile compounds at once (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008), was 97.7%.

3.2. Validation of the SPME-GC/QTOF method

3.2.1. Limits of detection (LODs) and quantification (LOQs)

For quantitative purposes, the method was performed in split 1:100 mode, as explained in Sub-section 2.7. The LODs ranged between $3.60\,\mu g\,Kg^{-1}$ and $1.76\,mg\,Kg^{-1}$ while the LOQs were between $12.0\,\mu g\,Kg^{-1}$ and $5.85\,mg\,Kg^{-1}$, although the average values were 213 and $709\,\mu g\,Kg^{-1}$, respectively (Table 1).

For semi-quantitative purposes, the method was performed in splitless mode, with LODs ranging between $0.801\,\mu g\,Kg^{-1}$ and $4.46\,\mu g\,Kg^{-1}$ (Table 1). The LODs were much lower because the samples were not diluted.

3.2.2. Precision: intra-day repeatability and inter-day repeatability

Taking results from the quantitative method into consideration, as they allow for drawing better conclusions, the % RSD for intra-day repeatability varied between 0.120% and 7.89%, while values for the inter-day repeatability were between 0.830% and 9.00% (Table 1). Thus, the quantitative method was deemed sufficiently precise with no need for an internal standard.

3.2.3. Linearity and accuracy

For quantification of the commercial sample, the MSA was applied. Almost all the volatile compounds were verified by the *t*-test and almost all the R^2 were higher than 0.99 (data not shown), except for acetic acid and furfuryl alcohol. Both acetic acid and furfuryl alcohol presented R^2 values lower than 0.99, probably due to a lack of homogeneity in the blank (crust sample without spiking). The accuracy exhibited % Re between 0.085 and 12.2%, with an average value of 2.47% (Table 1). Therefore, the method was declared accurate.

3.3. Quantification of volatile compounds of commercial wheat bread crust

The results, in $\mu g\,Kg^{-1}$, of the concentration of the 44 volatile compounds quantified in the commercial bread crust sample are shown in Table 2. The most abundant compounds, in decreasing order, were furfural, butyrolactone, acetoin, phenylethyl alcohol, phenylacetaldehyde, 3-methyl-1-butanol, pyrazine, 1-hexanol, 2-methyl-1-propanol and 2-acetylpyrrol, with concentrations higher than $1\,mg\,Kg^{-1}$. Furfural has been also reported by Raffo et al. (2015) as the most abundant compound, by far, of the volatile compounds of wheat bread crust analysed by SPME-GC/MS. Phenylacetaldehyde, phenylethyl alcohol, 3-methyl-1-butanol, 1-hexanol and 2-acetylpyrrol were also reported by Raffo et al. (2015) and Pacyński et al. (2015), who also analysed the volatile compounds of wheat bread crust as well as gluten-free bread crust by SPME-GC/MS.

3-Methyl-1-butanol has been reported as the main volatile compound in wheat bread crumb generated by fermentation (Birch, Petersen, & Hansen, 2014), thus its presence in the crust should be due to a migration from the crumb. Phenylethyl alcohol and phenylacetaldehyde are mainly generated in crumb by fermentation, but they can also be generated in the crust by Strecker degradation during Maillard reactions (Birch et al., 2014; Pico et al., 2015). On the contrary, furfural

Table 2

Concentration, in µg Kg^{-1}, of the 44 studied volatile compounds in the crusts of wheat, basmati rice, japonica rice, wheat starch and teff breads as well as in the commercial bread. Values are means of three determinations ± SD. Different letters in the same row show the significant differences, excluding the commercial crust. The numeration given in parentheses of each volatile compound corresponds to the number assigned in the corresponding PCA (Fig. 2).

Volatile compound	µg Kg^{-1} wheat flour crust	µg Kg^{-1} basmati rice crust	µg Kg^{-1} japonica rice crust	µg Kg^{-1} wheat starch crust	µg Kg^{-1} teff crust	µg Kg^{-1} commercial crust
2,3-Butanedione **(1)**	4.98 a ± 0.112	5.21 a ± 0.117	44.3 d ± 0.992	13.0 b ± 0.291	19.1 c ± 0.427	0.824 ± 0.427
Hexanal **(2)**	4.36 b ± 0.104	7.06 c ± 0.169	16.7 d ± 0.400	1.69 a ± 0.0404	17.1 d ± 0.409	0.907 ± 0.409
2-Methyl-1-propanol	8.93 b ± 0.0429	14.3 c ± 0.0686	71.4 e ± 0.343	1.09 a ± 0.00523	17.8 d ± 0.0852	1.04 ± 0.0852
1-Methylpyrrol **(3)**	1.40 b ± 0.00560	0.0700 a ± 0.000280	2.10 d ± 0.00840	2.13 d ± 0.00852	1.62 c ± 0.00648	0.386 ± 0.00648
Heptanal **(4)**	14.2 c ± 0.235	0.500 a ± 0.00825	20.6 e ± 0.340	16.9 d ± 0.279	6.06 b ± 0.100	0.903 ± 0.100
R-Limonene **(5)**	0.0100 a ± 0.0000780	11.5 e ± 0.0895	0.220 b ± 0.00172	1.49 c ± 0.0116	1.90 d ± 0.0148	0.118 ± 0.0148
Pyrazine **(6)**	4.21 b ± 0.0316	0.920 a ± 0.00690	7.70 d ± 0.0578	6.75 c ± 0.0506	9.77 e ± 0.0733	1.11 ± 0.0733
2-Methyl-1-butanol	7.49 c ± 0.431	3.32 a ± 0.191	5.29 b ± 0.304	4.81 ab ± 0.277	12.4 d ± 0.714	0.676 ± 0.714
3-Methyl-1-butanol **(7)**	10.5 c ± 0.417	8.63 b ± 0.342	10.7 c ± 0.424	11.1 c ± 0.438	6.94 a ± 0.275	1.56 ± 0.275
1-Pentanol	1.88 a ± 0.117	6.77 c ± 0.422	4.90 b ± 0.305	4.64 b ± 0.289	6.64 c ± 0.414	0.609 ± 0.414
2-Methylpyrazine **(8)**	14.0 d ± 0.0741	1.57 a ± 0.00832	19.8 e ± 0.105	11.5 b ± 0.0612	12.4 c ± 0.0657	0.871 ± 0.0657
Acetoin **(9)**	9.36 c ± 0.256	4.30 a ± 0.117	7.71 b ± 0.210	9.38 c ± 0.256	27.8 d ± 0.758	4.34 ± 0.758
2-Octanone **(10)**	21.8 d ± 0.983	0.640 a ± 0.0289	14.0 c ± 0.633	10.8 b ± 0.488	10.2 b ± 0.461	0.000100 ± 0.461
2,5-Dimethylpyrazine **(11)**	10.7 b ± 0.532	0.820 a ± 0.0406	38.1 d ± 1.89	23.2 c ± 1.15	0.790 a ± 0.0391	0.143 ± 0.0391
2,6-Dimethylpirazine **(12)**	4.70 c ± 0.175	0.760 a ± 0.0283	0.150 a ± 0.00560	11.6 d ± 0.431	2.01 b ± 0.0750	0.237 ± 0.0750
2-Ethylpyrazine	6.52 d ± 0.0437	0.860 c ± 0.00576	0.330 a ± 0.00221	8.09 e ± 0.0542	0.640 b ± 0.00429	0.358 ± 0.00429
2-Acetyl-1-pyrroline **(13)**	0.0459 e ± 0.000574	0.000210 a ± 0.00000263	0.00743 b ± 0.0000928	0.0321 c ± 0.000402	0.0398 d ± 0.000498	0.0146 ± 0.000221
2,3-Dimethylpyrazine	14.9 e ± 0.0744	0.820 a ± 0.00410	5.75 b ± 0.0288	11.3 d ± 0.0564	8.47 c ± 0.04235	0.255 ± 0.04235
1-Hexanol	15.4 c ± 0.117	0.320 a ± 0.00243	0.770 b ± 0.00585	0.700 b ± 0.00532	21.4 d ± 0.163	1.11 ± 0.163
Nonanal **(14)**	2.95 b ± 0.0758	0.640 a ± 0.0164	4.13 c ± 0.106	2.75 b ± 0.0707	4.21 c ± 0.108	0.0598 ± 0.108
2,3,5-Trimethylpyrazine **(15)**	0.730 a ± 0.00416	0.440 a ± 0.00251	37.1 c ± 0.212	48.1 d ± 0.274	28.9 b ± 0.165	0.0661 ± 0.165
2-Ethyl-3-methylpirazine **(16)**	0.420 a ± 0.00391	0.600 a ± 0.00558	26.8 b ± 0.249	0.460 a ± 0.00428	32.1 c ± 0.298	0.127 ± 0.298
Ethyl octanoate **(17)**	0.240 a ± 0.0129	0.0900 a ± 0.00485	3.83 c ± 0.206	0.690 b ± 0.0372	0.150 a ± 0.00809	0.360 ± 0.00809
1-Octen-3-ol **(18)**	4.48 b ± 0.160	0.600 a ± 0.0215	24.1 d ± 0.862	16.5 c ± 0.591	18.5 c ± 0.663	0.338 ± 0.663
Acetic acid **(29)**	nq[a]	nq[a]	nq[a]	nq[a]	nq[a]	nq[a]
Furfural	7.35 c ± 0.175	4.49 b ± 0.107	0.760 a ± 0.0181	26.1 d ± 0.622	1.10 a ± 0.0262	7.68 ± 0.0262
2-Ethyl-1-hexanol **(19)**	0.290 a ± 0.0150	0.440 a ± 0.0227	0.250 a ± 0.0129	17.7 b ± 0.913	0.740 a ± 0.0383	0.139 ± 0.0383
Benzaldehyde **(20)**	0.210 a ± 0.00174	0.340 a ± 0.00282	15.8 b ± 0.131	0.380 a ± 0.00315	0.320 a ± 0.00266	0.116 ± 0.00266
2-(E)-Nonenal **(21)**	0.970 d ± 0.0110	4.43 e ± 0.0501	0.210 b ± 0.00237	0.0400 a ± 0.000452	0.370 c ± 0.00418	0.0329 ± 0.00418
5-Methyl-2-furaldehyde	0.450 d ± 0.000585	0.0900 a ± 0.000117	0.150 b ± 0.000195	0.220 c ± 0.000286	0.500 e ± 0.000650	0.0357 ± 0.000650
Butyrolactone	9.39 c ± 0.415	17.2 d ± 0.762	6.71 ab ± 0.297	8.08 bc ± 0.357	6.15 a ± 0.272	6.48 ± 0.272
2-Acetilpyrazine **(22)**	12.0 c ± 0.152	0.0600 a ± 0.000762	4.97 b ± 0.0631	0.110 a ± 0.00140	0.130 a ± 0.00165	0.0799 ± 0.00165
Butyric acid **(23)**	5.28 c ± 0.0692	0.170 a ± 0.00223	8.08 e ± 0.106	2.17 b ± 0.0284	6.55 d ± 0.0858	0.283 ± 0.0858
Phenylacetaldehyde**(24)**	4.70 c ± 0.0620	2.50 b ± 0.0330	0.610 a ± 0.00805	1.08 a ± 0.0143	22.1 d ± 0.292	1.83 ± 0.292
Furfuryl alcohol **(30)**	nq[a]	nq[a]	nq[a]	nq[a]	nq[a]	nq[a]
2-Methylbutyric acid	3.45 b ± 0.190	1.64 a ± 0.0904	9.21 d ± 0.507	3.97 b ± 0.219	6.35 c ± 0.350	0.176 ± 0.350
3-Methylbutyric acid **(25)**	4.74 b ± 0.374	2.41 a ± 0.190	5.49 b ± 0.433	9.32 c ± 0.735	12.6 d ± 0.996	0.397 ± 0.996
2,4-(E,E)-Decadienal **(26)**	0.110 a ± 0.00572	0.350 b ± 0.0182	1.13 d ± 0.0588	0.630 c ± 0.0328	0.450 b ± 0.0234	0.0247 ± 0.0234
Hexanoic acid	13.0 c ± 0.0156	18.3 e ± 0.0219	15.0 d ± 0.0180	2.54 a ± 0.00305	10.8 b ± 0.0130	0.0246 ± 0.0130
Benzyl alcohol	0.290 a ± 0.00162	0.160 a ± 0.000896	34.4 b ± 0.193	0.220 a ± 0.00123	0.110 a ± 0.000616	0.104 ± 0.000616
Phenylethyl alcohol	13.0 b ± 0.510	5.54 a ± 0.217	56.5 d ± 2.21	2.02 a ± 0.0790	18.6 c ± 0.726	3.59 ± 0.726
2-Acetylpyrrol	4.38 b ± 0.169	0.450 a ± 0.0174	0.300 a ± 0.0116	27.6 c ± 1.06	5.97 b ± 0.230	1.02 ± 0.230
4-Hydroxy-2,5-dimethyl-3(2H)-furanone **(27)**	26.8 e ± 1.00	7.69 b ± 0.288	3.96 a ± 0.148	22.6 d ± 0.845	20.0 c ± 0.746	0.572 ± 0.746
4-Vinylguaiacol **(28)**	1.93 a ± 0.0720	13.0 c ± 0.486	4.16 b ± 0.155	2.39 a ± 0.0891	22.8 d ± 0.851	0.758 ± 0.851

[a] nq = not quantified.

is mainly generated in the crust by Maillard reactions (Jensen, Oestdal, Skibsted, Larsen, & Thybo, 2011; Martins, Jongen, & Van Boekel, 2000; Poinot et al., 2008, 2010) and caramelisation from 5-hydroxymethylfurfural (Ameur, Rega, Giampaoli, Trystram, & Birlouez-Aragon, 2008), although it can also be generated in the crumb during fermentation and then transferred to the crust (Birch et al., 2014; Pico et al., 2015). 1-Hexanol is mainly generated in the crumb by lipid oxidation and can be transferred to the crust. However, the hydroperoxides that bring about volatile compounds from lipid oxidation can also be broken in the crust, generating volatile compounds too (Moskowitz et al., 2012; Pico et al., 2015). Therefore, 2-acetylpyrrol is the only one generated exclusively by Maillard reactions (Poinot et al., 2010) in the crust.

In accordance with Moskowitz et al. (2012), the main compounds found in our samples generated in the crust have been products of Maillard reactions and lipid oxidation reactions. 2-acetyl-pyrroline, 4-hydroxy-2,5-dimethyl-3(2H)-furanone as well as 2-acetylpyrazine have been reported as the major active volatile compounds from Maillard reactions in wheat bread crust (Moskowitz et al., 2012; Schieberle & Grosch, 1985; Zehentbauer & Grosch, 1998), and 2-(E)-nonenal and 2,4-(E,E)-decadienal have reportedly served as the major active volatile compounds from lipid oxidation reaction (Moskowitz et al., 2012). Consequently, 2-acetyl-1-pyrroline and 2-(E)-nonenal have been selected as the most important aroma contributors due to their high dilution factor (FD) (Schieberle & Grosch, 1991) and their low odour thresholds (OT), which are 0.053 μg Kg^{-1} and 0.08 μg Kg^{-1}, respectively, in water. Although they were not the most abundant compounds in our samples, all of these volatile compounds have been quantified in the commercial wheat bread crust of the present study.

3.4. Selection of the gluten-free flours or starches in order to quantify the volatile compounds of the corresponding bread crust: semi-quantitative analyses

The volatile profile of five crusts of breads made with gluten-free flours (basmati rice, japonica rice, oat, teff and quinoa), two crusts of breads made with starches (corn and wheat) and the crust of wheat bread as a control sample, were studied in a semi-quantitative way in order to select the most suitable gluten-free flour or starch bread crust to be quantified (Table 3).

Fig. 1 shows the PCA of the 44 volatile compounds (labelled from1 to 44 in Table 3), in peak areas, examined in the seven gluten-free bread crusts and the wheat bread crust. With the aim of avoiding the highest areas to have more importance in the weight of the PCs, the three first PCs were normalised as a correlation matrix (Table S3), calculated as the PC multiplied by the square root of the corresponding eigenvalue. Only those compounds with normalised PCs higher than 0.700 were taken into consideration in the following discussion. Additionally, volatile compounds that typically appear in the crust with low OTs were considered in this section. Thus, the volatile compounds selected for this discussion were: pyrazine, 2-methylpyrazine, 2,5,-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2-ethyl-3-methylpyrazine, all from the Maillard reaction, and 2-(E)-nonenal, 2,4-(E,E)-decadienal and benzaldehyde, from lipid oxidation.

As an overview of the scores plot of the PCA, rice bread crust as well as teff bread crust were the samples with volatile profiles more similar to wheat bread crust, since they were nearer in the PCA. 4-Hydroxy-2,5-dimethyl-3(2H)-furanone and 2-(E)-nonenal were the volatile compounds that greatly contributed to the positive PC1 (see normalised PC1, Table S3) and have been reported as important contributors to bread crust aroma (Zehentbauer & Grosch, 1998). They were found in higher proportion in quinoa crust, wheat crust, teff crust and in lower proportion in rice crust, oat crust, basmati crust, corn starch crust and wheat starch crust. This could be the reason of the separation of rice crust from wheat and teff crust regarding the PC1. 2,4-(E,E)-decadienal, benzaldehyde and acetic acid presented also high values of normalised PC1 (Table S3) and they were found in highest proportion in quinoa crust, which explains its separation from the other crusts regarding the PC1. On the other hand, the negative PC1 was characterised by the highest contributions of pyrazines, including pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine and 2-ethyl-3-methylpyrazine, all with high values of normalised PC1 (Table S3). Wheat starch crust presented the highest proportions of all of them, being expected that this would lead to a darker crust colour, but it was one of the lightest crusts (data not shown). Pyrazines have been reported as important Maillard compounds in bread crust (Paraskevopoulou et al., 2012), that should contribute to its colour (Cho & Peterson, 2010); however, wheat flour crust was the darkest crust. This suggested that these pyrazines were not the responsible of the crust colour from compounds of Maillard reactions. Additionally, furan derivatives have been reported to contribute to the colour of the heated food (Hofmann, 1998). Concretely, furfuryl alcohol has been reported to polymerise in acidic conditions to aliphatic polymers that give a brown colouration to the bread (Okaru & Lachenmeier, 2017). Wheat flour crust presented the highest abundance of furfuryl alcohol, which can explain it darkest colour. Moreover, the similarity between wheat starch crust and wheat flour crust, most notably regarding the negative PC2, was related to the high content of pyrazines. Finally, the highest proportion of 2-ACPY, key aroma in the crust (Pico et al., 2015), was found in oat crust, although it was located opposite to wheat bread and its use was disregarded.

As a consequence, the crusts were distinguished mainly due to their content in pyrazines, 2-ACPY, 2-(E)-nonenal and 2,4-(E,E)-decadienal, as it was explained in Sub-section 3.3. Therefore, since rice crust and teff crust were located near to wheat crust, they were selected to be quantified. Due to the high content of pyrazines, wheat starch crust was also selected to be quantified and basmati crust was chosen in order to study the effect of other varieties of rice.

3.5. Quantification of the volatile compounds of the selected gluten-free bread crusts: improvement of gluten-free bread crust aroma

Forty-two volatile compounds from wheat starch bread crust as well as teff, japonica rice and basmati rice bread crusts were quantified (Table 2). Wheat bread crust served as a control sample and acetic acid and furfuryl alcohol were excluded from the quantification, since their R^2 values were lower than 0.99 and they did not pass the *t*-test for linearity. For a simplification in the interpretation of the results, only those volatile compounds with OTs lower than 1 mg Kg^{-1} were taken into consideration. This criterion was selected since only the volatile compounds with a concentration higher than their OTs have an important impact on the final aroma of bread. In this way, as all the volatile compounds were in a concentration lower than 1 mg Kg^{-1}, an OT lower than 1 mg Kg^{-1} was taken as a reference (Table S3). Then, the PCA was constructed with the concentration values, in μg Kg^{-1}, of the volatile compounds labelled in Table 2 from number 1 to 28 (Fig. 2). Moreover, the PCs normalised with the correlation matrix higher than 0.700 were considered important contributors.

Regarding the scores plot, basmati bread crust and wheat bread crust were located in the negative PC1 while wheat starch, rice and teff bread crusts were found in the positive PC1. In the negative PC1 of the loadings plot there were only 4 volatile compounds, which meant that both breads presented a less complex volatile profile in the crust. Basmatic crust was characterised by the highest content in 2-(E)-nonenal and limonene while wheat crust was characterised by the highest content in 4-hydroxy-2,5-dimethyl-3(2H)-furanone (similar to the content in wheat starch). The content of 2-(E)-nonenal in wheat crust was the second highest, almost 3 times higher than the third one (teff crust). Thus, the main difference between wheat crust and rice, teff and wheat starch crusts should be found in the content of 2-(E)-nonenal. There are some controversies about the impact of 2-(E)-nonenal on the final aroma of bread, since it has been reported as correlating positively with green notes (Hansen & Hansen, 1996; Salim-

Table 3

Peak areas, divided by 10^6, of the 44 studied volatile compounds in the crusts of corn starch, wheat starch, basmati rice, japonica rice, oat, teff, quinoa and wheat breads as well as in the commercial bread. Values are means of three determinations ± SD. Different letters in the same row show the significant differences. The numeration given in parentheses of each volatile compound corresponds to the number assigned in the corresponding PCA (Fig. 1).

Volatile compound	Corn starch crust	Wheat starch crust	Basmati rice crust	Japonica rice crust	Oat crust	Teff Crust	Quinoa crust	Wheat flour crust
2,3-Butanedione **(1)**	0.876 ab ± 0.0449	0.826 ab ± 0.0991	1.11 b ± 0.0983	0.991 b ± 0.0987	0.605 a ± 0.0710	0.919 ab ± 0.231	0.960 b ± 0.251	1.05 b ± 0.138
Hexanal **(2)**	3.78 a ± 0.112	6.31 e ± 0.535	4.76 b ± 0.362	7.46 f ± 0.408	5.84 de ± 0.294	5.37 bcd ± 0.200	5.66 cde ± 0.140	5.11 bc ± 0.0208
2-Methyl-1-propanol **(3)**	0.100 ab ± 0.0115	0.190 d ± 0.00213	0.183 cd ± 0.0209	0.252 e ± 0.0346	0.0613 a ± 0.00648	0.252 e ± 0.0375	0.0611 a ± 0.00857	0.141 bc ± 0.00215
1-Methylpyrrol **(4)**	0.0374 a ± 0.00513	0.116 c ± 0.0123	0.102 c ± 0.00139	0.0658 b ± 0.00300	0.0476 ab ± 0.00512	0.160 d ± 0.0208	0.124 c ± 0.0139	0.116 c ± 0.00460
Heptanal **(5)**	0.949 a ± 0.0531	1.41 bc ± 0.0771	1.08 ab ± 0.0489	2.22 d ± 0.0214	1.23 ab ± 0.0316	3.17 f ± 0.178	2.65 e ± 0.124	1.74 c ± 0.355
R-Limonene **(6)**	0.416 a ± 0.0570	2.82 c ± 0.214	1.88 b ± 0.311	2.32 c ± 0.314	0.773 a ± 0.216	1.88 b ± 0.232	2.54 c ± 0.334	2.36 c ± 0.291
Pyrazine **(7)**	2.62 bc ± 0.0211	3.60 d ± 0.132	3.59 d ± 0.296	3.02 c ± 0.115	2.26 b ± 0.216	2.38 b ± 0.284	0.636 a ± 0.0489	3.01 c ± 0.0482
2-Methyl-1-butanol **(8)**	1.38 a ± 0.218	3.30 e ± 0.186	2.53 cd ± 0.191	2.84 d ± 0.295	1.01 a ± 0.0368	2.06 b ± 0.224	1.04 a ± 0.0546	2.19 bc ± 0.218
3-Methyl-1-butanol **(9)**	2.63 b ± 0.254	3.89 d ± 0.342	3.81 cd ± 0.0323	3.75 cd ± 0.0501	2.28 ab ± 0.113	3.50 c ± 0.0440	2.16 a ± 0.000333	3.65 cd ± 0.132
1-Pentanol **(10)**	0.216 a ± 0.0319	0.793 bc ± 0.258	0.799 bc ± 0.0745	1.80 d ± 0.305	1.07 c ± 0.0309	0.731 b ± 0.0450	1.07 c ± 0.0225	0.817 bc ± 0.00182
2-Methylpyrazine **(11)**	0.856 c ± 0.00643	2.14 f ± 0.00514	1.26 e ± 0.0352	1.09 d ± 0.00524	1.22 e ± 0.00658	1.15 d ± 0.0351	0.158 a ± 0.00877	0.773 b ± 0.0589
Acetoin **(12)**	4.43 ab ± 0.0937	4.18 a ± 0.172	5.19 c ± 0.0738	4.64 b ± 0.0573	5.74 d ± 0.231	4.61 b ± 0.0342	6.32 e ± 0.176	5.97 de ± 0.291
2-Octanone **(13)**	0.542 ab ± 0.0680	1.42 e ± 0.0662	0.629 c ± 0.0212	0.894 d ± 0.0256	0.366 a ± 0.00951	1.05 d ± 0.150	1.33 e ± 0.137	1.02 d ± 0.140
2,5-Dimethylpyrazine **(14)**	0.185 b ± 0.0118	0.715 f ± 0.0414	0.338 d ± 0.00568	0.289 c ± 0.0211	0.667 e ± 0.00713	0.358 d ± 0.0166	0.0432 a ± 0.00512	0.179 b ± 0.0199
2,6-Dimethylpirazine **(15)**	0.190 b ± 0.0154	0.299 d ± 0.00149	0.186 b ± 0.00286	0.184 b ± 0.00158	0.416 e ± 0.0129	0.199 bc ± 0.00468	0.0622 a ± 0.00162	0.221 c ± 0.138
2-Ethylpyrazine **(16)**	0.0941 b ± 0.00348	0.258 f ± 0.00484	0.157 d ± 0.00361	0.152 d ± 0.00768	0.157 d ± 0.00767	0.210 e ± 0.00940	0.0442 a ± 0.00424	0.129 c ± 0.0208
2-Acetyl-1-pyrroline **(17)**	0.772 c ± 0.0411	1.07 d ± 0.0759	0.690 bc ± 0.105	0.318 a ± 0.00103	1.37 e ± 0.0309	0.366 a ± 0.0878	0.498 ab ± 0.0838	0.317 a ± 0.0518
2,3-Dimethylpyrazine **(18)**	3.55 b ± 0.00348	5.81 e ± 0.0737	3.65 b ± 0.262	3.48 b ± 0.0415	5.13 d ± 0.0641	3.53 b ± 0.215	0.817 a ± 0.0294	4.18 c ± 0.00460
1-Hexanol **(19)**	0.591 a ± 0.0934	1.46 c ± 0.212	1.29 c ± 0.129	2.81 d ± 0.106	0.918 b ± 0.0738	3.66 e ± 0.203	3.95 e ± 0.0109	1.53 c ± 0.355
Nonanal **(20)**	1.64 a ± 0.296	1.57 a ± 0.691	1.31 a ± 0.281	1.83 a ± 0.401	1.80 a ± 0.239	1.72 a ± 0.497	1.93 a ± 0.401	1.75 a ± 0.291
2,3,5-trimethylpyrazine **(21)**	0.111 b ± 0.00363	0.567 f ± 0.0370	0.162 c ± 0.0150	0.144 bc ± 0.00613	0.333 e ± 0.00146	0.200 d ± 0.000373	0.0445 a ± 0.00381	0.127 b ± 0.0482
2-Ethyl-3-methylpirazine **(22)**	1.84 b ± 0.0624	4.90 g ± 0.0361	2.20 c ± 0.102	2.44 cd ± 0.187	3.48 e ± 0.242	3.80 f ± 0.128	0.777 a ± 0.0109	2.61 d ± 0.218
Ethyl octanoate **(23)**	2.88 ab ± 0.458	4.40 cd ± 0.485	3.34 bc ± 0.517	3.12 ab ± 0.482	1.97 a ± 0.235	4.75 d ± 0.317	4.05 bcd ± 0.840	3.32 bc ± 0.132
1-Octen-3-ol **(24)**	0.0523 c ± 0.0000341	0.0293 ab ± 0.00329	0.0319 b ± 0.00522	0.0323 b ± 0.00702	0.0214 a ± 0.00223	0.0500 c ± 0.00724	0.028 ab ± 0.00446	0.0270 ab ± 0.00182
Acetic acid **(25)**	0.961 a ± 0.114	1.62 bc ± 0.0788	1.55 bc ± 0.130	1.49 b ± 0.126	1.49 b ± 0.0580	2.06 d ± 0.178	2.98 e ± 0.286	1.88 cd ± 0.131
Furfural **(26)**	0.436 b ± 0.0367	0.437 b ± 0.0255	0.482 bc ± 0.0248	0.541 c ± 0.0185	0.232 a ± 0.00130	2.27 e ± 0.0704	4.58 f ± 0.0714	2.17 d ± 0.0272
2-Ethyl-1-hexanol **(27)**	0.598 b ± 0.0629	0.602 b ± 0.113	0.536 ab ± 0.0442	0.562 b ± 0.0388	0.515 ab ± 0.00605	0.421 a ± 0.00181	0.425 a ± 0.00735	0.535 ab ± 0.00725
Benzaldehyde **(28)**	0.0514 a ± 0.00504	0.083 ab ± 0.00903	0.138 bc ± 0.0216	0.141 bc ± 0.00224	0.101 abc ± 0.0123	0.122 abc ± 0.00890	0.166 c ± 0.0710	0.147 bc ± 0.0536
2-(E)-Nonenal **(29)**	0.0519 bc ± 0.00477	0.023 a ± 0.00167	0.0473 b ± 0.00677	0.0630 c ± 0.00108	0.0463 b ± 0.00374	0.0796 d ± 0.00711	0.0853 d ± 0.00922	0.0619 c ± 0.00744
5-Methyl-2-furaldehyde **(30)**	0.0719 a ± 0.00133	0.088 a ± 0.000283	0.0860 a ± 0.00667	0.0896 a ± 0.00323	0.0760 a ± 0.00461	0.735 c ± 0.0229	1.89 d ± 0.0363	0.414 b ± 0.00920
Butyrolactone **(31)**	2.59 b ± 0.308	2.08 ab ± 0.409	2.08 ab ± 0.117	1.79 a ± 0.249	2.59 b ± 0.221	4.40 d ± 0.381	4.01 cd ± 0.175	3.54 c ± 0.151
2-Acetilpyrazine **(32)**	0.0986 a ± 0.00782	0.131 ab ± 0.0320	0.233 cd ± 0.0284	0.140 ab ± 0.0339	0.268 d ± 0.0189	0.198 bc ± 0.0334	0.0953 a ± 0.000390	0.246 cd ± 0.0487
Butyric acid **(33)**	0.0507 a ± 0.00442	0.527 b ± 0.0572	0.0515 a ± 0.00144	0.0586 a ± 0.00337	0.0329 a ± 0.00164	0.0550 a ± 0.00586	0.0500 ± 0.000718	0.0460 a ± 0.00252
Phenylacetaldehyde **(34)**	1.58 e ± 0.174	1.55 e ± 0.0777	0.739 cd ± 0.00331	0.917 d ± 0.0896	0.321 a ± 0.0467	0.662 bc ± 0.0554	0.507 ab ± 0.0534	0.430 a ± 0.0339
Furfuryl alcohol **(35)**	0.906 c ± 0.0472	1.08 d ± 0.0303	0.845 bc ± 0.00720	0.922 c ± 0.00571	0.538 a ± 0.0101	0.753 b ± 0.0505	2.15 e ± 0.0782	3.24 f ± 0.0649
2-Methylbutyric acid **(36)**	2.08 a ± 0.323	3.25 b ± 0.677	2.99 ab ± 0.485	3.22 b ± 0.663	2.01 a ± 0.283	3.31 b ± 0.439	2.42 ab ± 0.217	3.38 b ± 0.351
3-Methylbutyric acid **(37)**	2.29 ab ± 0.374	2.94 bc ± 0.518	2.90 bc ± 0.545	3.16 c ± 0.421	1.73 a ± 0.153	3.35 c ± 0.229	2.67 bc ± 0.171	3.36 c ± 0.270
2,4-(E,E)-Decadienal **(38)**	0.0381 a ± 0.00138	0.147 b ± 0.0198	0.0395 a ± 0.00303	0.152 b ± 0.0195	0.0432 a ± 0.00147	0.0521 a ± 0.00427	0.525 c ± 0.0568	0.0602 a ± 0.00822
Hexanoic acid **(39)**	0.0312 ab ± 0.000241	0.147 c ± 0.00238	0.0543 ab ± 0.00689	0.0831 b ± 0.00375	0.0249 a ± 0.00144	0.445 e ± 0.0637	0.271 d ± 0.0201	0.0480 ab ± 0.000601
Benzyl alcohol **(40)**	0.853 a ± 0.250	1.20 a ± 0.338	1.44 a ± 0.284	8.01 c ± 0.664	4.66 b ± 0.431	1.070 a ± 0.261	4.91 b ± 0.356	0.827 a ± 0.104
Phenylethyl alcohol **(41)**	0.772 a ± 0.0742	1.79 c ± 0.108	1.62 c ± 0.0223	1.20 b ± 0.115	0.653 a ± 0.0478	1.10 b ± 0.0265	0.806 a ± 0.0570	2.02 c ± 0.183
2-Acetylpyrrol **(42)**	2.28 b ± 0.165	1.68 a ± 0.237	2.44 b ± 0.0961	2.57 bc ± 0.260	2.55 bc ± 0.160	3.44 d ± 0.209	4.31 e ± 0.267	2.94 c ± 0.00399
4-Hydroxy-2,5-dimethyl-3(2H)-furanone **(43)**	0.0484 a ± 0.0127	0.0238 a ± 0.00371	0.0853 ab ± 0.00224	0.0719 ab ± 0.00324	0.116 bc ± 0.0102	0.160 c ± 0.000359	0.495 e ± 0.0652	0.289 d ± 0.0424
4-Vinylguaiacol **(44)**	0.108 cd ± 0.0154	0.0200 a ± 0.000557	0.102 bcd ± 0.00686	0.0688 abc ± 0.00524	0.0487 ab ± 0.00266	0.155 d ± 0.00776	0.765 e ± 0.0661	0.0714 abc ± 0.00766

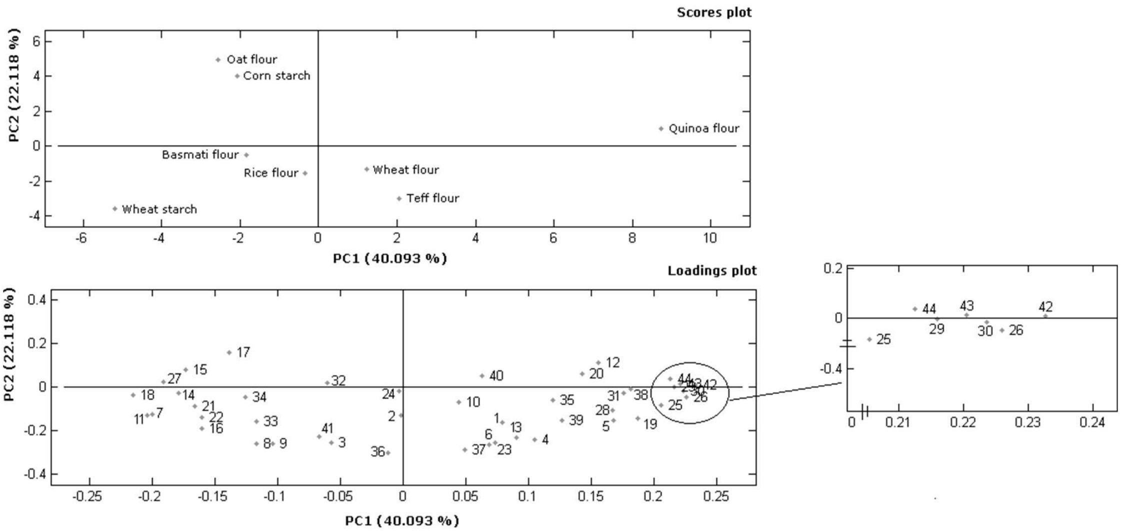

Fig. 1. PCA of the gluten-free bread crusts and wheat bread crust (control sample) analysed semi-quantitatively by SPME-GC/QTOF (peak areas represented). The scores plot represents the 8 samples and the loadings plot of the 30 volatile compounds selected for presenting low odour thresholds. The numbers corresponding to each volatile compound are indicated in Table S3.

ur-Rehman, Paterson, & Piggott, 2006), but also negatively with fatty notes (Quílez, Ruiz, & Romero, 2006). In fact, it has been reported as one of the volatile compounds responsible for the staling of bread (Zehentbauer & Grosch, 1998). Regarding the negative notes generated during the staling of bread, the lower concentration of 2-(E)-nonenal in wheat starch, teff and rice crusts could be considered a positive attribute for gluten-free breads. Finally, although it contributed minimally to the correlation matrix (Table S3), 4-hydroxy-2,5-dimethyl-3(2H)-

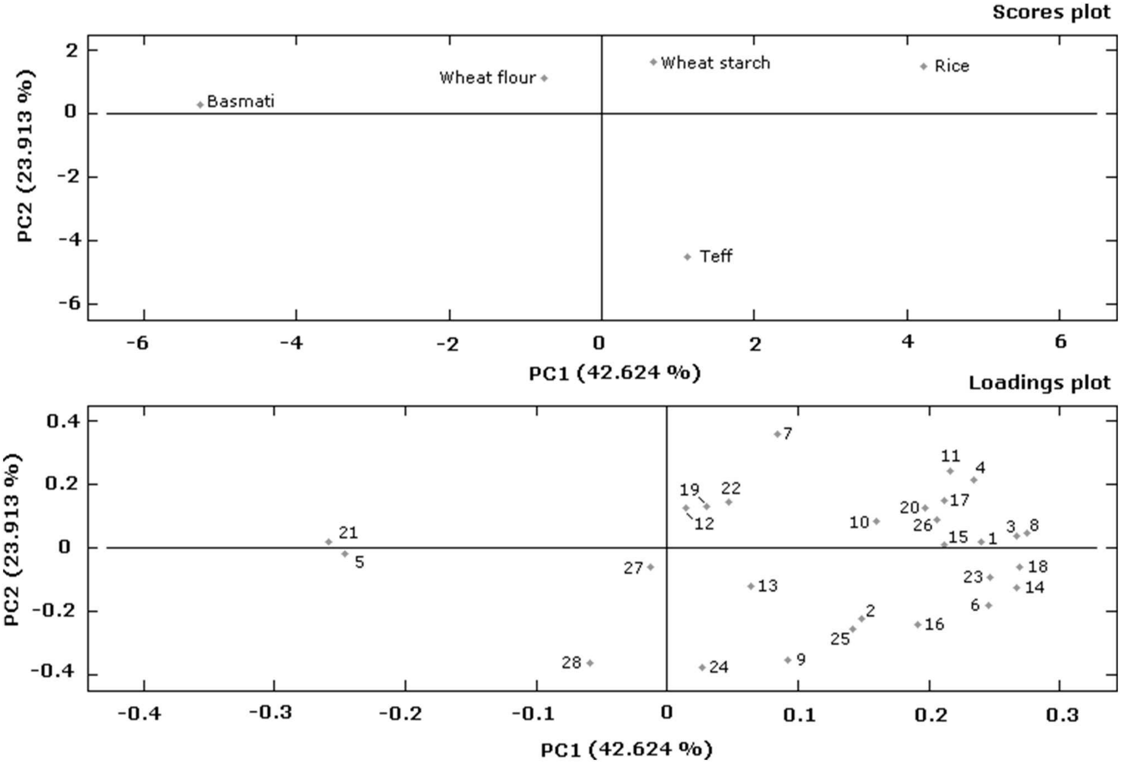

Fig. 2. PCA of the gluten-free bread crusts and wheat bread crust (control sample) analysed quantitatively by SPME-GC/QTOF (concentrations represented in μg Kg^{-1}). The scores plot represents the 5 samples and the loadings plot of the 28 volatile compounds quantified. The numbers corresponding to each volatile compound are indicated in Table S3.

furanone from Maillard reactions (Moskowitz et al., 2012) has been reported as an important contributor to crust aroma (Zehentbauer & Grosch, 1998), with a caramel-like smell (Moskowitz et al., 2012).

In the positive PC1 of the loadings plot, 1-octen-3-ol, pyrazine, 2-methylpyrazine and 2,3,5-trimethylpyrazine were the volatile compounds in highest abundance; they were common for the rice, wheat starch and teff bread crusts. Moreover, all of them presented correlation values higher than 0.75, thus they contributed to the overall flavour of the crust. 1-Octen-3-ol is a volatile compound from lipid oxidation that has been reported to correlate negatively with the final aroma of bread (Paraskevopoulou et al., 2012), presenting the highest concentration in the rice bread crust. Although the content of lipids is higher in teff than in rice and wheat starch (USDA Database, 2009), the amount of lipoxygenases is higher in rice (Wongdechsarekul & Kongkiattikajorn, 2010) and the concentration of antioxidants, such as flavonoids and vitamin E, is lower in rice (Inglett, Chen, & Liu, 2015). Then, the oxidation of lipids is encouraged in rice crust, justifying the highest amount of 1-octen-3-ol in rice crust (lower amount of lipids but higher lipoxygenase activity and lower antioxidant action) and in the second place in teff crust (higher amount of lipids but lower lipoxygenase activity and higher antioxidant action). In fact, rice crust and teff crust presented similar amounts of hexanal and nonanal, volatile compounds of lipid oxidation (Pico et al., 2015), which explained the balance between the content of lipids and the amount of lipoxygenases and antioxidants. The three pyrazines were in similar concentrations in rice, wheat starch and teff bread crusts, which could be one of the reasons for their separation from basmati and wheat crusts. 2,3,5-Trimethylpyrazine was the most abundant pyrazine overall and, in wheat starch crust, it was in highest concentration. In general, pyrazines have been reported as important Maillard compounds in bread crust (Paraskevopoulou et al., 2012), contributing greatly to its colour (Cho & Peterson, 2010). The darkest crust was that of teff, followed by rice and then wheat starch (data not shown), but the concentration of these pyrazines was not very different (see Table 2). This suggested, as in the semi-quantitative section, that pyrazine, 2-methylpyrazine and 2,3,5-trimethylpyrazine were not responsible for crust colour. The same reasoning could be applied to the highest content of 2,6-dimethylpyrazine in wheat starch crust.

Within the positive PC1, teff crust (negative component of the PC2) was separated from rice and wheat starch crust (positive component of the PC2). Higher contents of heptanal, 2,4-decadienal, 1-methylpyrrol and 2,5-dimethylpyrazine found in rice and wheat starch crusts compared to teff crust could explain this separation; meanwhile, teff was characterised by the highest content in fermentation volatile compounds like acetoin, phenylacetaldehyde and 3-methylbutanoic acid. However, these three fermentation volatile compounds did not show high values of correlation (Table S3), probably because their presence depended on the migration from the crumb to the crust and not on their homogeneous generation in the crust. The same occurred with the highest content of 3-methyl-1-butanol in wheat starch crust, which had a content similar to that of wheat flour crust. Heptanal and 2,4-decadienal, with correlation values higher than 0.70, are lipid oxidation volatile compounds (Birch et al., 2014) with the highest concentration in rice, probably due to the same reasons explained for 1-octen-3-ol. 1-Methylpyrrol as well as 2,5-dimethylpyrazine, both volatile compounds from the Maillard reaction (Poinot et al., 2008), had high values of correlation, although they have not been reported as important contributors to the crust aroma.

Therefore, the gluten-free bread crusts were mainly distinguished by their contents of volatile compounds from lipid oxidation and Maillard reactions, which have been reported as the main compounds in the crust of wheat bread (Moskowitz et al., 2012), as it was explained in Sub-section 3.4. For wheat bread crust, the control sample, the most abundant volatile compound was 4-hydroxy-2,5-dimethyl-3(2H)-furanone (26.8 μg Kg^{-1}). This could be the reason for the similarity between wheat flour crust and teff and wheat starch crusts, as the contents of 4-hydroxy-2,5-dimethyl-3(2H)-furanone were 20.0 μg Kg^{-1} and 22.6 μg Kg^{-1}, respectively. Moreover, the contents of the key aroma 2-ACPY in wheat flour bread crust and in teff and wheat starch bread crusts were also similar (0.0459 μg Kg^{-1}, 0.0398 μg Kg^{-1} and 0.0321 μg Kg^{-1}, respectively), explaining the likeness of teff and wheat starch crusts regarding wheat flour crust. In the case of teff crust, 2-ACPY was the most abundant compound followed by 2-ethyl-3-methylpyrazine (32.1 μg Kg^{-1}), while in the case of wheat starch crust 2,3,5-trimethylpyrazine was the most abundant (48.1 μg Kg^{-1}) and 2-ACPY was second in abundance. Therefore, a suitable mixture between wheat starch and teff flour was suggested in order to improve the final aroma of gluten-free bread.

By contrast, Pacyński et al. (2015) reported that their gluten-free breads were characterised by a lack of pyrazines and 2-acetyl-1-pyrroline compared to the control wheat bread. However, we found contents of pyrazines that varied between 0.440 and 48.1 μg Kg^{-1} among the four gluten-free breads and contents of 2-ACPY that varied between 0.210 and 39.8 μg Kg^{-1}. The differences are surprising since Pacyński et al. (2015) added, besides corn and wheat starches, sources of amino acids and sugars that encourage the Maillard reaction, like glucose, milk powder and egg. Therefore, it would be expected that pyrazines and other compounds from Maillard reaction were found in the gluten-free breads studied by Pacyński et al. (2015).

4. Conclusions

A SPME-GC/QTOF methodology for the analyses of 44 volatile compounds in bread crusts has been developed, optimised and validated. The optimisation was accomplished with the application of the Response Surface Method (RSM), with previous reduction of the dimensionality employing Principal Component Analysis (PCA). The final SPME conditions were 0.75 g of crust extracted at 60 °C for 51 min. The SPME-GC/QTOF methodology was validated in terms of LOD and LOQ, precision, accuracy and linearity, proving that it was sensible, precise, accurate and linear. The methodology was applied to quantification through the Method of Standard Addition (MSA) of a commercial wheat bread crust. Furfural, which comes from Maillard reactions and caramelisation processes, was the most abundant compound in the commercial wheat flour bread crust, corresponding with the literature. Four selected gluten-free bread crusts (rice, basmati, teff and wheat starch) were also quantified and compared with a wheat bread crust control sample. It was concluded that wheat starch crust as well as teff crust were the closest to the control wheat crust due to their similar contents in 2-acetyl-1-pyrroline (2-ACPY), 4-hydroxy-2,5-dimethyl-3(2H)-furanone and pyrazines, which have been reported as main compounds in wheat bread crust.

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Appendix A. Supplementary data

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IX. Section 5

Study of the most appropriate mixture of flours and starches for the improvement of the final aroma of gluten-free bread

Selection of the most suitable mixture of flours and starches for the improvement of gluten-free breads through their volatile profiles

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Highlights

- ✓ Higher corn starch content increased the rancid volatiles from lipid oxidation.
- ✓ Higher wheat starch content decreased the amount of 2-acetyl-1-pyrroline.
- ✓ Equal proportions of each starch (40%) provided the most pleasant bread aroma.
- ✓ Increase too much quinoa and teff flours content did not give special aroma notes.
- ✓ Wheat bread (control) showed the lowest abundance of almost all the volatiles.

Abstract

Gluten-free breads have been characterised by a weak aroma. In this study, different mixtures of gluten-free flours (teff and quinoa) and starches (wheat and corn) were investigated with the aim of improving the aroma of gluten-free bread using SPME-GC/QTOF. The bread with the highest content of corn starch (60 %) was discarded for its higher proportion in rancid volatile compounds from lipid oxidation, while the bread with the highest content in wheat starch (60 %) was discarded for its lower level of 2-acetyl-1-pyrroline, a key aroma of the crust. Thus, the bread composed of 40 % of each starch, 15 % of quinoa flour and 5 % of teff flour was chosen due to the higher ratio of pleasant volatiles from fermentation, 2-acetyl-1-pyrroline and pyrazines as well as the lower ratio in rancid volatile compounds from lipid oxidation. Moreover, it exhibited relatively dark crust, high volume and superior textural properties.

Keywords: gluten-free bread; volatile compounds; SPME-GC/QTOF; physical properties.

Abbreviations: 2-ACPY (2-acetyl-1-pyrroline); CAR (carboxen); DVB (divinylbenzene); GC/QTOF (gas chromatography/quadrupole-time of flight); HPMC (hydroxyl propyl methyl cellulose); KI (Kovats Index); OT (odour threshold); PC (principal component); PCA (principal component analysis); PDMS (polydimethylsiloxane); PFTE (polytetrafluoroethylene) ; SPME (solid-phase microextraction).

1. Introduction

Gluten-free products, but above all gluten-free breads, have been characteristically very poor in quality. Gluten is considered an essential structure-building protein, contributing to the appearance, crumb structure, and consumer acceptance of wheat bread (Arendt, Morrissey, Moore, & Dal Bello, 2008). Peer-reviewed literature on gluten-free bread making has primarily focused on overall efforts to study and improve quality parameters such as nutritional values, rheology of the dough, texture, volume, colour or staling (Alvarez-Jubete, Arendt, & Gallagher, 2010; Houben, Höchstötter, & Becker, 2012; Mancebo, Merino, Martínez, & Gómez, 2015; Masure, Fierens, & Delcour, 2016; Pruska-Kędzior et al., 2008). Several possibility have emerged as means to increase the physical characteristics of gluten-free breads: increasing their water-binding capacity, homogenising the crumb structure, increasing the final bread volume, achieving a less crumbly texture or darkening the crust by using different gluten-free flours and starches, hydrocolloids and gums, proteins, enzymes, fats, oils and emulsifiers (Houben et al., 2012). However, there is little knowledge regarding the analysis of the aroma gluten-free bread. There is some information regarding sensorial analyses (Masure et al., 2016), but which volatile compounds were the responsible of the preference for a particular gluten-free bread were not specified. For this purpose, it is necessary to analyse the volatile profiles of the gluten-free breads. Until now, research in gluten-free bread aroma has focused on understanding the origin of the volatile compounds compared to wheat bread (Poinot et al., 2009), the improvement of gluten-free bread aroma based on the method of baking (Aguilar et al., 2015) or the improvement of the crust aroma with the addition of sugar-amino acid pairs to encourage the Maillard reaction (Pacyński et al., 2015). Therefore, it is necessary to develop gluten-free bread formulas that look for not only good physical parameters but also good aroma (Pacyński et al., 2015).

Arendt et al. (2008) recommended the use of a range of gluten-free flours rather than just one flour to achieve products with good sensory and textural properties. Moreover, they reported that the addition of a certain percentage of starch improved the overall quality of the gluten-free bread, which was in concordance with the findings reported by Mancebo et al. (2015). However, none of these articles studied the volatile profiles of the bread as well as the effect of mixing flours and starches on the final aroma of bread. It is our belief that in order to select the suitable proportion of each flour and starch in the gluten-free bread formulation for the improvement of its aroma, it is necessary to first know the individual influence of each flour and starch. For this purpose, our research group studied the individual influence of different flours and starches on the aroma of the crumb (Pico, Bernal, & Gómez, 2017; Pico, Hansen, & Petersen, 2017) and crust (Pico et al., 2018). Quinoa flour and corn starch were selected for their abilities to enhance the aroma of the crumb, and it was concluded that the use of quinoa increased the proportion of pleasant volatile compounds from fermentation while corn starch increased the volatile 2,3-butanedione; both presented low levels of rancid volatile compounds from lipid oxidation. Teff flour and wheat starch were selected for their improvement of the aroma of the crust, since both increased the contents of 2-acetyl-1-pyrroline and pyrazines. However, the effect of teff in the aroma of the crumb was not very positive since it increased the content of rancid volatile compounds from lipids oxidation. Moreover, the use of quinoa should be limited due to its trace content of bitter saponins. On the other hand, high contents of starches could lead to breads with crusts that are excessively light and crumbly. Subsequently, it is necessary to study the suitable proportion between both starches (wheat, corn) and flours (quinoa, teff) that improves the final aroma of gluten-free bread, while maintaining good physical properties.

Therefore, the aim of this project has been to find the most suitable mixture of gluten-free flours (quinoa, teff) and starches (wheat, corn) that improve the final aroma of gluten-free bread. Moreover, the selected mixture should present good physical parameters such as specific volume, moisture loss, hardness, springiness, cohesiveness, resilience and colour.

2. Materials and methods

2.1. Materials and standards

2-acetyl-1-pyrroline (2-ACPY) was purchased from Eptes (Vevey, Switzerland) and the other pure standards labelled from 1 to 43 in Table S1 were obtained from Sigma-Aldrich (Steinheim, Germany). Dichloromethane was obtained from Scharlab (Barcelona, Spain) and methanol from VWR International (Fontenay-sous-Bois, France). All the standards presented a purity at least of 99%. Argon, nitrogen and helium were acquired from Carburos Metálicos (Barcelona, Spain).

2.2. Preparation of standard solutions

2-ACPY solutions were prepared in dichloromethane, as 2-ACPY is only stable in dichloromethane and ethyl acetate. It was necessary to work under inert atmosphere of argon at all times due to the lack of stability of the compound to oxygen and moisture. For this reason, dichloromethane was dried in a SDS PS-MD-5 purification system from Düperthal Sicherheitstechnik (Karlstein am Main, Germany).

For the other 43 volatile compounds included in Table 1, working solutions of each volatile compound were prepared in methanol. All the solutions were stored in a freezer at -21 °C.

2.3. Gluten-free breads ingredients

Wheat starch was supplied by Roquette Laisa (Valencia, Spain) and corn starch by Miwon Daesang (Seoul, Republic of Korea). Wheat flour was purchased from Harinera Castellana (Medina del Campo, España), quinoa flour from El Granero Integral (Madrid, Spain) and teff flour from Salutef (Palencia, Spain). Hydroxyl propyl methyl cellulose (HPMC) was supplied by Dow Chemicals (Michigan, USA) and the dry baker's yeast (*Saccharomyces cerevisiae*) by Lesaffre (Cerences, France). All yeasts belonged to the same batch to decrease the risk of different cell count.

2.4. Gluten-free bread making

The following ingredients, as % of mixtures, were used in all the formulas: sunflower oil (6%), sucrose (5%), salt (1.8%), instant yeast (3%), HPMC (2%) and water (100%). Four breads prepared with different mixtures of flours (teff, quinoa) and starches (wheat, corn), as well as the wheat bread (control sample), were elaborated with this recipe. The proportions of the flours and starches in each mixture are shown in Table 1. All the ingredients were mixed using a Kitchen-Aid Professional mixer (KPM5, Kitchen Aid, St. Joseph, Michigan, USA) for 8 min at speed 2. 100 g of dough were fermented for 90 min in a chamber at 30 °C with 90% of humidity. The doughs were baked at 190 °C for 40 min. After baking, the gluten-free breads were left at room temperature for 30 min. In order to avoid problems with the homogeneity of the sample, the whole bread (including crumb and crust) was cut in small pieces, frozen with liquid nitrogen and ground. Each sample was prepared in duplicate (n=2).

2.5. Specific volume and moisture loss

Bread properties were evaluated 24 h after baking. Bread volume was determined using a laser sensor with the Volscan Profiler (Stable Micro Systems, Godalming, United Kingdom). Specific volume was calculated as the ratio of bread volume to its mass. Moisture loss (%) was calculated as the weight difference of the bread before and after the baking process. The volume and moisture loss measurements were performed on two loaves from each type of bread.

2.6. Texture parameters

Crumb texture was measured with a TA-XT2 texture analyser (Stable Microsystems, Surrey, UK) equipped with the "Texture Expert" software. A 25-mm diameter cylindrical aluminium probe was used in a "Texture Profile Analysis" (TPA) doublecompression test to penetrate up to 50% of the sample depth at a test speed of 2 mm/s, with a 30 s delay between the two compressions. Hardness, springiness, cohesiveness and resilience were calculated from the TPA curve. Texture analyses were performed on 20 mm thick central slices. Analyses were performed on two slices from two separate loaves for each formulation.

2.7. Colour

Bread colour was measured using a Minolta CN-508i spectrophotometer (Minolta Co., Ltd, Japan) with the D65 standard illuminant and the 2º standard observer and results were expressed in the CIE L*a*b* colour space. Colour measurements were made on four random points of the crust of two breads (4x2) and on two points of the crumb of two slices of two loaves (2x2x2) for each type of bread.

2.8. Solid-phase microextraction (SPME)

The method employed was previously optimised and validated previously by the research group (Pico, Antolín, Román, Gómez, & Bernal, 2018) for the analysis of volatile compounds in bread crust. 0.75 g (± 0.0050 g) of each bread sample (including crumb and crust, see sub-section 2.4) was weighed into a 20 mL vial and sealed with a magnetic screw cap with PTFE/silicone septa. The selected fibre was 50/30 µm DVB/CAR/PDMS of 1 cm of lenght (Sigma Aldrich, Gillingham, UK). The sample was incubated for 5 min at 60 °C (without the fibre) and then extracted for 51 min at 60 °C, without agitation. After that, the fibre was inserted into the GC injector port for thermal desorption for 5 min at 270 °C. Finally, the fibre was conditioned for 30 min at 270 °C after each analysis. Each bread sample (four mixtures and wheat bread) was analysed in triplicate (n=3).

2.9. GC/QTOF chromatographic conditions

GC/QTOF analyses were performed on a 7890A gas chromatograph coupled to a 7200 Quadrupole-Time of flight (QTOF) mass spectrometer detector and MassHunter B.07.00 software, all from Agilent Technologies (Santa Clara, California, USA). The GC was equipped with a CombiPAL RSI 85 autosampler from

CTC Analytics AG (Zwingen, Switzerland). The chromatographic conditions were previously optimised by the research group using standard solutions (Pico et al., 2018). The separation was achieved on a polar Innowax column (100% polyethylene glycol, 30 m × 0.25 mm ID × 0.25 µm) obtained from J&W Scientific (Agilent Technologies, California, USA). The GC was operated under programmed temperature conditions: from 45 °C (1.5 min) to 100 °C (0 min) at 7 °C/min, then the temperature was increased to 114 °C (6.7 min) at 1 °C/min, afterwards it was increased to 136 °C (0 min) at 2.5 °C/min and finally it was increased to 245 °C (5 min) at 85 °C/min. Total run time per sample was 43 min. The carrier gas was helium at a flow rate of 1.1 mL/min. The injector temperature was 270 °C, working in splitless mode for the less abundant volatile compounds and in split mode for the most abundant volatile compounds (see Table S1). The use of two working modes for different compounds was possible because the same volatile compound was followed among the different samples, and all samples were injected in the same mode. However, different compounds injected in different modes were not compared. The interface, ion source and quadrupole temperatures were 250°C, 230 °C and 150°C, respectively. Analyses were performed in SCAN mode and included a mass range of 20–350 m/z, operating in electron ionization mode with energy of 70 eV. All the volatile compounds labeled from 1 to 44 in Table S1 were identified by comparison of their retention times and accurate mass spectra (with four decimal places) with standards as well as by using their Kovats Index (KI) and Mass Spectra Library (NIST MS Search 2.2 & MS Interpreter). Those labeled from 45 to 52 were identified using their KI and Mass Spectra Library.

2.10. Statistical analysis

In order to detect significant differences in the analysed parameters between the bread samples (four mixtures and wheat bread), one-way Anova was computed by the software Statgraphics Centurion version XVII (Statpoint Technologies, Warrenton, Virginia, United States). In order to assess the variation of the volatile compounds among the different gluten-free breads, a PCA was conducted with the average peak area of each bread sample prepared in duplicate and analysed in triplicate (n=6). The PCA was performed with the software LatentiX version 2.00 (Latent5, Copenhagen, Denmark), with data standardized prior to the analysis.

3. Results and discussion

3.1. Physical properties of breads

The effect of the flour / starch source on the specific volume, moisture loss and crumb texture of breads is shown in Table 3. Breads made with higher amount of flour (wheat bread and mixture 1) had lower specific volume than those made with higher proportion of starch (mixture 2, 3 and 4, see also Figure 1). In fact, other authors have reported that the inclusion of starches generally increased bread volume when using mixtures of starches and gluten-free flours (Mancebo et al., 2015; Mariotti et al., 2013, Onyango et al., 2011). This effect is related, at least partially, to the higher consistency of flour-based doughs that could hinder dough expansion during baking (Martinez & Gómez, 2017). The low specific volume found for wheat bread can be explained by the presence of HPMC used in that formulation, which together with the gluten network could have given rise to a stiffer structure, hindering dough expansion. Another plausible mechanism is that HPMC may have competed for water with gluten, not allowing complete gluten hydration, and, hence, preventing the development of a correct gluten network. Nevertheless, the selected formulation for wheat bread is not typically used for gluten breads, since we used HPMC and higher hydrations. This formulation was employed in order to make the volatile profiles of gluten-free breads and wheat breads comparable. Regarding moisture loss, wheat bread presented lower moisture loss, which would be in agreement with the stiffer structure described above. No significant differences were found for this parameter in samples containing high amounts of starch (i.e., mixture 2, 3 and 4).

For the textural parameters, hardness values indicated breads with a stiffer crumb had lower volume (wheat bread and mixture 1). This negative relationship between specific volume and hardness has already been found in other studies (Gallagher et al., 2003; Martínez & Gómez, 2017; Martinez, Roman & Gomez, 2018). In the case of the breads with higher specific volume, the lowest hardness was found for breads made with higher amount of wheat starch (mixtures 2 and 4). This effect would be related to the better packing properties in these breads and the capacity of the dough of wheat starch to form a uniform continuous starch-hydrocolloid matrix, due to the bimodal size distribution of its starch granules. This was already suggested by Martinez and Gomez (2017) who found that the use of wheat starch in gluten-free bread resulted in breads with the highest specific volume and the best textural properties in terms of lower hardness and higher elasticity, cohesiveness and resilience. In fact, this would be in agreement with the results of our study, since despite not finding significant differences for springiness in any of the samples, higher values of springiness, cohesiveness and resilience were found for mixtures 2 and 4 compared to the other gluten-free counterparts (mixture 1 and 3); furthermore, slightly similar values were observed compared to wheat bread. The lower values of springiness and cohesiveness of the crumb have represented one of the main problems of gluten-free bread in relation to wheat bread (Moore, Schober, Dockery & Arendt, 2004). Therefore, the mixtures 2 and 4, with higher content in wheat starch, would be preferable in this regard.

3.2. Colour measurements

Crust colour was affected by the type of mixture as seen in Table 4. In general, the use of starches in the formulation brought about lighter (higher L* values) and less red crusts (lower a* values) with minimal differences in the yellow (b*) values. The brighter and less coloured crust observed in starch based breads may be related to the lower content of protein in these formulations (Smak, 1972), which would contribute to a lower extent of Maillard reactions responsible for colour development, and, hence, leading to paler crusts. The excessively pale crust colour in gluten-free breads in comparison to wheat breads is a problem that can be minimized with the use of flours, which present higher content of proteins (Gallagher et al., 2003; Mancebo et al., 2015). In this way, mixture 1, with the lowest content of starches, exhibited a darker crust, with values of L* and a* most similar to the wheat bread.

On the other hand, the colour of the crumb is usually associated with the colour of the initial main ingredients (starches and flours) used, since Maillard reactions do not take place in the crumb. In this way, darker crumbs with higher a* and b* values (red and yellow, respectively) were found for breads made with mixture 1 (also see Figure 1). The higher percentage of quinoa and teff flours in this mixture, which are darker in colour compared to starches and wheat flour, would have given rise to these colour attributes. Meanwhile, no significant differences were found for mixtures 2, 3 and 4, and their crumbs were only slightly less yellow compared to the wheat bread.

3.3. Volatile profiles of the different gluten-free breads and their relationship with physicochemical parameters

3.3.1. Selection of the gluten-free flours and starches

As it was previously explained, quinoa flour and corn starch were selected for the improvement of the aroma of the crumb (Pico, Bernal, et al., 2017; Pico, Hansen, et al., 2017), while teff flour and wheat starch were chosen for the enhancement of the aroma of the crust (Pico et al.2018). It was expected that mixtures of both flours and starches would improve the final aroma of the gluten-free bread. Firstly, a mixture with the same proportion of each flour/starch (mixture 1, Table 1) was tested as the starting point for gluten-free breads. Proportions higher than 5 % of teff flour were not tried since they would increase the content of unpleasant volatile compounds from lipid oxidation in crumb, while proportions higher than 15 % of quinoa flour were not tried either due to the bitterness provided by the traces of saponins. Taking this into consideration, mixture 2 was composed of the same proportion of each starch (40 %) in order to maintain the same relevance in the improvement of the crumb with the corn starch and in the improvement of the crust with the wheat starch. In order to understand the effect of a large increase of each starch (up to 60 %), mixtures 3 and 4 were tried and compared with mixture 2 where the proportion of each starch was the same.

3.3.2. Main differences in the volatile profiles of the gluten-free breads and wheat bread

Although 52 volatile compounds were detected, only those volatile compounds labeled from 1 to 44 (table S1) were taken into consideration, since they were confirmed with standards, KI and spectral library and they have been reported as important contributors to the final aroma of bread (Birch, Petersen, & Hansen, 2014). Those compounds labelled from 45 to 52 were identified by KI and spectral library. Regarding the scores plot of the PCA (Figure 1), there was a clear separation between the wheat bread and the four gluten-free breads. Specifically, the only compounds that were highest in proportion in wheat bread were acetoin, benzaldehyde, furfuryl alcohol and 2-methylbutanoic acid (Table 2). Furfuryl alcohol was the only compound with low odour threshold (OT) (Table S1), while acetoin was reported as positively correlated with the final aroma of bread (Pico, Bernal, & Gómez, 2015) due to its buttery notes (Table S1). Benzaldehyde has been reported to correlate negatively with the final aroma of wheat bread (Quílez, Ruiz, & Romero, 2006) due to its bitter almond notes, while 2-methylbutanoic acid, with higher OT than the 3-methyl isomer, has been reported to have a potential positive impact on the final aroma of wheat bread (Grosch, & Schieberle, 1997) with cheesy characteristics (Table S1).

The other 40 volatile compounds were in higher proportion in the gluten-free breads, which means that the powerful flavour that has been reported for wheat bread (Pacyński, Wojtasiak, & Mildner-Szkudlarz, 2015b) should be related to the proportion between the different volatile compounds and not with the highest concentration for all the volatile compounds. Moreover, the structure of bread should determine how the volatile compounds are released (Le Bail, Biais, Pozo-Bayón, & Cayot, 2004) and, therefore, how they are perceived by the consumer. Concretely, wheat bread showed the lowest specific volume as well as the lowest moisture loss (Table 3). As it has been explained in sub-section 3.1, the formulation selected for wheat bread, with HPMC and high hydrations, is not the common one for gluten breads and this could modify the volatile profile. However, it was necessary to use the same recipe for all the breads in order to know the influence of the flour/starch. Taking our wheat bread into consideration, the more compact the bread is, the more difficult the release of the volatile compounds to the atmosphere before being eaten should be. Then, if the release of volatile compounds in wheat bread is lower than for gluten-free breads, there is the possible hypothesis that the volatile compounds would remain in the bread matrix until they are released in the mouth at the moment of chewing. This could justify the stronger flavour of the wheat bread (Aguilar et al., 2015). As it has been explained in subsection 3.1, it is also related to hardness, because if there is a high resistance to the crumb deformation there would be less space for bubbles and less possibility for volatile compounds to be released (note that hardness was highest in wheat bread). Both hypotheses are in concordance with the lower moisture loss observed, since the easier evaporation of water would release more volatile compounds to the atmosphere that are able to interact with water, that is to say, those volatile compounds that are of hydrophilic nature.

3.3.2.1. Differences in the volatile compounds from fermentation and lipid oxidation

The bread marked as mixture 1 (25% of each flour and starch) is located in the y-axis (Figure 2), thus its contribution to the PC1 (54.6% of the variance) was negligible, since the value of this principal component for this sample is practically zero and its variability regarding this component is not clear. Therefore, its behavoiur is better explained by the PC2,whichc explained lower percentage of the total variability (21.9% of the variance). The volatile profile of mixture 1 was not characterised by the highest level of any volatile compound. Most of the volatile compounds were in higher proportion in mixtures 2, 3 and 4, which contained higher content of starches. As the mixture 1 presented the lowest content of starches and the highest content of flours, it can be concluded that the starches lead to interesting differences in the corresponding volatile profile.

On the other hand, mixtures 2 and 4 (both containing high quantitites of wheat starch, 40 % and 60 % respectively) were separated regarding the PC2 from mixture 3 (which contained the highest content of corn starch, 60 %, and low content of wheat starch, 25 %), mainly due to their ratio in volatile compounds from fermentation and lipid oxidation. As it can be seen in the loadings plot (Figure 1), mixtures 2 and 4 were located in the positive PC1/ negative PC2 due to their higher proportion in fermentation volatile compounds such as 2-methyl-1-propanol, 2/3-methyl-1-butanol, butyric acid, 2/3-methylbutanoic acid or acetic acid (Table 2). Mixture 3 was located in the positive PC1/ positive PC2 due to its higher level in lipid oxidation volatile compounds like hexanal, heptanal, 1-octen-3-ol, 2-(E)-nonenal, 2,4-(E,E)-decadienal or 2-octanone (Table 2). Among all of them, 3-methyl-1-butanol, 2-methyl-1-butanol, 3-methylbutanoic, 2-methylbutanoic (Pico et al., 2015) and acetic acid (Quílez et al., 2006) have been reported to have a potential positive impact in the final aroma of bread, although they present high OTs (Table S1). On the other hand, hexanal, 1-octen-3-ol, 2-(E)-nonenal, 2,4-(E,E)-decadienal (Pico et al., 2015) have been reported to correlate negatively with the final aroma of bread, due to its fatty and green notes (Table S1). Moreover, all of them presented low OTs (Table S1), and, above all, 2-(E)-nonenal and 2,4-(E,E)-decadienal whose OT were under 0.1 µg Kg^{-1}.

However, there are also some volatile compounds from fermentation that were in higher proportion in mixture 3, such as ethyl octanoate and phenylethyl alcohol, and some volatile compound from lipids oxidation, notably 1-pentanol, 1-hexanol and nonanal, that were in higher level in mixture 2 and 4. Both ethyl octanoate and phenylethyl alcohol have been reported to exhibit pleasant aromas, fruity and rose respectively (Table S1). Among the lipid oxidation volatile compounds higher in mixtures 2 and 4, only nonanal presented low OT with unpleasant fatty notes (Table 1). The occurrence of these volatile compounds mainly affected the aroma of the crumb (Birch et al., 2014), although there could have been transferences from the crumb to the crust (Onishi, Inoue, Araki, Iwabuchi, & Sagara, 2011). At any event, the major occurrence of volatile compounds from lipid oxidation that correlated negatively with the final aroma of bread led to the conclusion that mixture 3 should be disregarded in terms of the crumb aroma.

3.3.2.2. Differences in the volatile compounds from Maillard reactions

Interesting differences should be found in the volatile compounds that came from the Maillard reactions, which should have mainly affected the aroma of the crust. All the pyrazines, pyrroles and furan derivatives as well as 2-ACPY were located in the positive PC1, which means that they were in higher proportion in the gluten-free breads than in the wheat bread. The higher ratio of volatile compounds from Maillard reactions in gluten-free breads was in concordance with prior results obtained by the research group for the analyses of the volatile profiles of crusts of different gluten-free breads (Pico et al. 2018). On the contrary, Pacyński et al. (2015) reported that their gluten-free breads were characterised by a lack of pyrazines and 2-ACPY compared to wheat bread. The differences were unexpected since Pacyński et al. (2015) added, apart from starches, sources of amino acids and sugars that promote the Maillard reaction, such as glucose, milk powder and egg. The analytical technique employed by Pacyński et al. (2015) was SPME-GC/MS with CAR/PDMS as a fibre, while we employed SPME-GC/QTOF with DVB/CAR/PDMS. The selected fibre influences the adsorption of certain volatile compounds (Pauline Poinot et al. 2007), and we also studied the influence of the fibre in the volatile profile of the wheat bread crust and found that 2-acetyl-1-pyrroline, acetylpyrazine and 2-ethy-3-methylpyrazine were 1.5, 1.5 and 2.7 times higher for DVB/CAR/PDMS than for CAR/PDMS, respectively (Pico et al. 2018).

Although Maillard compounds have been reported to correlate positively with the crust colour, the four gluten-free breads presented lighter crusts compared to the wheat bread (Cho & Peterson, 2010). Thus, as it was explained in subsection 3.2, the colour of the different crusts was measured and the results were given in the CIE L*a*b* system (Table 4). For the crust, the wheat bread presented a L* of 50.8, while for the mixtures 1, 2, 3 and 4 L* were 61.4, 74.5, 78.0 and 76.9, respectively. Then, mixture 1 showed the darkest crust but it was not considered in the aroma discussion since it was in the y-axis of the PCA. Mixture 2 and mixture 4 (40 % and 60 % of wheat starch, respectively) were the next darker, without significant differences between them, which indicates that the increase of wheat starch did not change the colour. However, when the proportion of corn starch was increased from 40 % (mixture 2) to 60% (mixture 3), the crust became the lightest. On the other hand, the level of 2-ACPY in mixture 3 was constant compared to mixture 2, while in the mixture 4 the proportion of 2-ACPY decreased relative to mixture 2 (Table 2). This suggests that the increase in the content of corn starch had no effect in the concentration of 2-ACPY, while the increase in the content of wheat starch implied a decrease in the percentage of 2-ACPY. In any case, all the mixtures presented higher proportion of 2-ACPY than wheat bread. The last has been considered the key aroma of wheat flour bread crust (Zehentbauer & Grosch, 1998), due to its very low odour threshold (OT) of 0.053 and its roasted, popcorn aroma. Conversely, when the percentage of corn starch was increased from 40 % in mixture 2 to 60 % in mixture 3, the ratio of pyrazine, 2,5-dimethylpyrazine, 2-ethyl-3-methylpyrazine and 2,3,5-trimethylpyrazine remained steady. It can be concluded that the increase in the proportion of corn starch did not involve changes in the concentration of pyrazines either. However, when wheat starch content was increased in mixture 4, the fraction of 2-methylpyrazine, 2-ethylpyrazine, 2,6-dimethylpyrazine and 2,3-dimethylpyarazine rose relative to mixture 2. As a consequence, it can be concluded that 2-ACPY and the studied pyrazines were not responsible for the colour of the crust, since they were in higher proportion than in wheat bread but the crust was lighter. Supporting this conclusion, the decrease in the colour with the increase of corn starch (from mixture 2 to mixture 3) did not involve changes in the percentage of 2-ACPY and the studied pyrazines, while the decrease of the level of 2-ACPY with the increase of wheat starch was not related to the preservation of the colour (from mixture 2 to mixture 4). Finally, although the studied pyrazines have been reported to exhibit pleasant aroma with nutty, chocolate and earthy notes, they presented medium OT (Table S1). Only 2-ethyl-3-methylpyrazine presented an OT of 0.4 µg Kg^{-1} but the odour was burnt nutty. Therefore, as the concentration of 2-ACPY decreased and only the portion of some pyrazines increased compared to mixture 2, mixture 4 was disregarded in terms of the crust aroma.

Furfural, 5-methyl-2-furaldehyde and furfuryl alcohol are furan derivatives that are generated by Maillard reactions but can be also generated by caramelisation processes (Ait Ameur, Rega, Giampaoli, Trystram, & Birlouez-Aragon, 2008). Specifically, furfural is generated by the reaction between phenylalanine and xylose (Nakama, Kim, Shinohara, & Omura, 2014). On its part, 5-methyl-2-furaldehyde is derived from phenylalanine and rhamnose (Buera, Chirife, Resnik, & Wetzler, 1987). Regarding caramelisation, both furfural and 5-methyl-2-furaldehyde are mainly produced by pentose degradation or even 5-hydroxymethylfurfural (Ait Ameur et al., 2008). Furfuryl alcohol is mainly reported as a reduction product from furfural (Spillman, Pollnitz, Liacopoulos, Pardon, & Sefton, 1998). Furfuryl alcohol polymerises in acidic conditions to aliphatic polymers that give a brown colouration to the bread (Okaru & Lachenmeier, 2017). Specifically, furfuryl alcohol was the only product of Maillard reactions that was in higher abundance in wheat bread, instead of in gluten-free breads (Table 2). Considering its polymerisation to the aliphatic polymers, furfuryl alcohol could be one of the compounds responsible for the dark crust of wheat bread (Table 4). Moreover, furfuryl alcohol was the only one of the three compounds with low OT and pleasant notes (Table S1). Furfural and 5-methyl-2-furaldehyde presented pleasant sweet and almond characteristics but their OT was notably high.

4. Conclusions

Four different mixtures of flours and starches were tested in order to improve the final aroma of gluten-free bread. Based on previous studies, teff flour and wheat starch were selected for the improvement of the crust and quinoa flour and corn starch for the improvement of the crumb. The bread with highest content in corn starch (mixture 3) was dismissed for its high ratio of volatile compounds from lipid oxidation (important in crumb), and the bread with the highest content of wheat starch (mixture 4) was discarded for its low level of 2-ACPY and pyrazines (important in crust). Although the volatile profile is different from the wheat bread in accordance with the PCA, the bread with 40 % of each starch (mixture 2) was selected as the most suitable due to its aroma characteristics, with higher proportion in pleasant volatile compounds from fermentation, such as 3-methyl-1-butanol, lower proportion in unpleasant volatile compounds from lipids oxidation, such as hexanal, but, above all, with a very high levels in 2-acetyl-1-pyrroline and pyrazines, even higher than in the wheat bread. Moreover, mixture 2 presented one of the darkest crusts and, due to the high content of wheat starch, very good textural characteristics. On its part, wheat bread was characterised only by the highest level of acetoin, benzaldehyde, furfuryl alcohol and 2-methylbutanoic acid. This suggested that the powerful aroma of wheat bread should be related to the proportion between the different volatile compounds and not to the highest concentration in all of them. Additionally, the lowest ratio of 2-acetyl-1-pyrroline and pyrazines in wheat bread, which presented the darkest crust, suggested that these volatile compounds were not the responsible for the colour of the crust.

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Table 1. Formulations followed for making the gluten-free breads as well as the wheat bread (control sample). The weight of each ingredient is given in g/100g.

Ingredients (g)	**Mixture 1**	**Mixture 2**	**Mixture 3**	**Mixture 4**	**Wheat bread**
Wheat starch	25.0	40.0	25.0	60.0	* nu
Corn starch	25.0	40.0	60.0	25.0	* nu
Quinoa flour	25.0	15.0	10.0	10.0	* nu
Teff flour	25.0	5.00	5.00	5.00	* nu
Wheat flour	* nu	* nu	* nu	* nu	100
Tap water	100	100	100	100	100
Sunflower oil	6.00	6.00	6.00	6.00	6.00
Sugar (sacarose)	5.00	5.00	5.00	5.00	5.00
Salt (NaCl)	1.80	1.80	1.80	1.80	1.80
HPMC	2.00	2.00	2.00	2.00	2.00
Dry baker’s yeast	3.00	3.00	3.00	3.00	3.00

* nu = not used

Table 2. Volatile compounds found in the four gluten-free breads as well as in the wheat bread (control sample). Values are given as peak areas divided by 10^6 and they are means of three determinations ± RSD. Values followed by the same letters within each parameter indicate no significant differences in the one-way ANOVA test (p>0.05).

Volatile compounds	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Wheat bread
2,3-Butanedione	1.32 a ± 0.170	3.24 c ± 0.116	3.35 c ± 0.00906	1.84 b± 0.150	1.04 a ± 0.0429
Hexanal	0.321 a ± 0.0297	0.788 c ± 0.0123	0.903 d ± 0.00731	0.653 b ± 0.00686	0.365 a ± 0.0202
2-Methyl-1-propanol	0.538 cd ± 0.0113	0.589 d ± 0.0180	0.422 b ± 0.0388	0.489 c ± 0.0117	0.274 a ± 0.0183
1-Methylpyrrol	0.282 cd ± 0.00219	0.226 bc ± 0.0167	0.348 d ± 0.0731	0.152 ab ± 0.00519	0.105 a ± 0.00703
Heptanal	0.227 c ± 0.00652	0.199 b ± 0.0136	0.277 d ± 0.00443	0.174 a ± 0.00472	0.218 bc ± 0.00482
R-Limonene	1.77 b ± 0.0194	2.16 c ± 0.190	2.94 d ± 0.0982	1.35 a ± 0.0195	1.26 a ± 0.0685
Pyrazine	0.784 c ± 0.0368	0.900 d ± 0.0142	1.17 e ± 0.0411	0.529 a ± 0.00158	0.614 b ± 0.0195
2-Methyl-1-butanol	0.169 b ± 0.00446	0.186 b ± 0.00297	0.171 b ± 0.00365	0.219 c ± 0.00620	0.130 a ± 0.0144
3-Methyl-1-butanol	0.320 b ± 0.00365	0.351 c ± 0.00498	0.346 c ± 0.00541	0.412 d ± 0.00103	0.150 a ± 0.00207
1-Pentanol	1.56 b ± 0.00447	2.47 d ± 0.00262	2.43 d ± 0.0477	1.76 c ± 0.0182	0.959 a ± 0.0502
2-Methylpyrazine	0.842 b ± 0.00561	1.18 c ± 0.00759	1.24 d ± 0.0169	1.25 d ± 0.00967	0.483 a ± 0.0334
2-Octanone	2.63 b ± 0.151	2.90 c ± 0.0496	3.03 c ± 0.0825	2.61 b ± 0.0480	0.885 a ± 0.0107

Table 2. (Continued)

Volatile compounds	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Wheat bread
Acetoin	0.180 a ± 0.00190	0.200 b ± 0.00431	0.238 c ± 0.000947	0.245 c ± 0.00255	0.452 d ± 0.0131
2,5-Dimethylpyrazine	0.767 b ± 0.0269	0.988 d ± 0.00338	0.990 d ± 0.00100	0.901 c ± 0.0206	0.357 a ± 0.00730
2,6-Dimethylpyrazine	0.297 b ± 0.00140	0.317 bc ± 0.000976	0.339 c ± 0.0152	0.500 d ± 0.00858	0.270 a ± 0.0109
2-Ethylpyrazine	0.270 b ± 0.000440	0.344 c ± 0.00744	0.332 c ± 0.00278	0.382 d ± 0.00551	0.154 a ± 0.00817
2-ACPY	0.523 ± 0.0215	0.774 ±0.0326	0.778 ± 0.0389	0.582 ± 0.0289	0.371 ± 0.0112
2,3-Dimethylpyrazine	0.0715 a ± 0.00118	0.111 c ± 0.000919	0.115 d ± 0.000217	0.143 e ± 0.000681	0.0924 b ± 0.000337
1-Hexanol	0.136 d ± 0.00242	0.103 b ± 0.00107	0.0827 a ± 0.00310	0.143 e ± 0.00198	0.110 c ± 0.000175
Nonanal	1.02 b ± 0.00792	1.02 b ± 0.00567	1.06 b ± 0.0399	1.24 c ± 0.0112	0.332 a ± 0.00420
2,3,5-trimethylpyrazine	0.215 b ± 0.00581	0.339 d ± 0.00986	0.350 d ± 0.00680	0.274 c ± 0.0150	0.175 a ± 0.00122
2-Ethyl-3-methylpyrazine	0.697 b ± 0.00866	0.854 d ± 0.00144	0.867 d ± 0.00533	0.763 c ± 0.0180	0.384 a ± 0.000957
Ethyl octanoate	0.759 c ± 0.0233	0.770 c ± 0.0366	0.833 d ± 0.0224	0.613 b ± 0.00582	0.421 a ± 0.00366
1-Octen-3-ol	0.351 b ± 0.0106	0.453 c ± 0.00582	0.454 c ± 0.0126	0.372 b ± 0.00933	0.252 a ± 0.00938
Acetic acid	2.27 c ± 0.0565	2.15 b ± 0.0122	2.49 d ± 0.0198	2.62 e ± 0.0460	1.70 a ± 0.0101

Table 2. (Continued)

Volatile compounds	**Mixture 1**	**Mixture 2**	**Mixture 3**	**Mixture 4**	**Wheat bread**
Furfural	2.81 b ± 0.207	2.58 ab ± 0.0173	3.06 c ± 0.0510	2.73 b ± 0.0127	2.85 a ± 0.0135
2-Ethyl-1-hexanol	0.339 b ± 0.00415	0.439 c ± 0.0102	0.426 c ± 0.00374	0.328 b ± 0.0163	0.232 a ± 0.00453
Benzaldehyde	0.831 b ± 0.0115	0.885 c ± 0.00639	1.01 d ± 0.0174	0.791 a ± 0.00545	1.64 e ± 0.0188
2-(E)-Nonenal	0.0569 a ± 0.00223	0.109 d ± 0.00551	0.157 e ± 0.00210	0.0954 c ± 0.00311	0.0816 b ± 0.000526
5-Methyl-2-furaldehyde	0.345 b ± 0.0208	0.259 a ± 0.0242	0.318 b ± 0.00775	0.250 a ± 0.0136	0.231 a ± 0.00354
Butyrolactone	3.15 d ± 0.0255	2.27 bc ± 0.152	2.38 c ± 0.0855	2.02 a ± 0.0122	2.15 ab ± 0.0542
2-Acetylpyrazine	0.648 c ± 0.00614	0.513 b ± 0.0116	0.696 d ± 0.00410	0.490 b ± 0.0146	0.150 a ± 0.00141
Butyric acid	0.191 b ± 0.00248	0.312 c ± 0.00736	0.300 c ± 0.0111	0.610 d ± 0.00749	0.117 a ± 0.000821
Phenylacetaldehyde	1.21 b ± 0.0264	2.26 c ± 0.0964	2.92 e ± 0.0348	2.63 d ± 0.00742	0.579 a ± 0.0220
Furfuryl alcohol	0.528 a ± 0.0311	0.683 ab ± 0.00715	0.891 c ± 0.0100	0.811 c ± 0.0258	1.23 d ± 0.196
2-Methylbutanoic acid	0.0876 a ± 0.00055	0.114 b ± 0.00292	0.157 c ± 0.000338	0.163 d ± 0.00396	0.182 e ± 0.000814
Phenylethyl alcohol	4.42 a ± 0.212	6.28 c ± 0.288	7.41 d ± 0.138	4.87 ab ± 0.292	5.28 b ± 0.378

Table 2. (Continued)

Volatile compounds	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Wheat bread
3-Methylbutanoic acid	0.109 a ± 0.00100	0.130b ± 0.000794	0.153 c ± 0.000535	0.188 d ± 0.00120	0.155 c ± 0.00451
2,4-(E,E)-Decadienal	0.433 b ± 0.0197	0.660 c ± 0.0362	0.882 d ± 0.0206	0.429 b ± 0.00189	0.332 a ± 0.0163
Hexanoic acid	0.479 b ± 0.0406	0.513 bc ± 0.0211	0.545 cd ± 0.00400	0.601 d ± 0.0144	0.188 a ± 0.00881
Benzyl alcohol	0.183 e ± 0.00123	0.152 c ± 0.00142	0.156d ± 0.000429	0.125 b ± 0.000190	0.0568 a ± 0.0000432
2-Acetylpyrrol	0.255 c ± 0.0168	0.189 b ± 0.00133	0.234 c ± 0.00903	0.161 a ± 0.00455	0.170 ab ± 0.00669
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	0.0764 d ± 0.00114	0.0567b ± 0.00342	0.0819e ± 0.00121	0.0507a ± 0.000981	0.0661 c ± 0.000256
4-Vinylguaiacol	0.495 c ± 0.0126	0.516 c ± 0.0200	0.728 d ± 0.0119	0.324 b ± 0.0282	0.177 a ± 0.00206
3-Methylbutanal	3.41 b ± 0.0548	3.42 b ± 0.0197	3.53 b ± 0.00222	3.54 b ± 0.0653	2.48 a ± 0.323
2-Pentylfuran	1.28 b ± 0.110	1.51 c ± 0.0424	1.66 c ± 0.0500	1.16 b ± 0.0340	0.765 a ± 0.0241
Octanal	3.99 c ± 0.106	4.81 d ± 0.161	5.53 e ± 0.389	3.45 b ± 0.149	1.04 a ± 0.0147
Ethyl heptanoate	3.46 d ± 0.229	2.73 c ± 0.108	2.27 b ± 0.123	2.18 b ± 0.196	0.224 a ± 0.0445
2-Ethyl-5-methylpyrazine	0.589 b ± 0.0296	0.768 cd ± 0.0373	0.802 d ± 0.0157	0.711 c ± 0.0250	0.367 a ± 0.0135
3-Furaldehyde	2.57 a ± 0.161	2.33 a ± 0.153	2.81 a ± 0.103	2.47 a ± 0.0508	2.56 a ± 0.495
Ethyl nonanoate	5.96 d ± 0.371	3.92 c ± 0.0802	2.99 b ± 0.0512	2.41 b ± 0.337	0.744 a ± 0.0612
Ethyl decanoate	3.92 d ± 0.120	3.77 d ± 0.202	3.31 c ± 0.0983	2.41 b ± 0.215	1.69 a ± 0.0893

Table 3. Physicochemical parameters of the different gluten-free breads as well as the wheat bread (control sample). Values are means of three determinations ± RSD and the composition of the breads is indicated in Table 1. Values followed by the same letters within each parameter indicate no significant differences in the one-way ANOVA test (p>0.05).

Bread	**Specific Volume (ml/g)**	**Moisture loss (g/100g)**	**Hardness (N)**	**Springiness**	**Cohesiveness**	**Resilience**
Wheat	6.49 a ± 0.0149	28.6 a ± 0.303	2.41 c ± 2.33	0.99 a ± 0.01	0.790 c ± 0.0146	0.402 c ± 0.000105
Mixture 1	6.93 b ± 0.000923	36.9 c ± 0.0522	2.30 c ± 1.24	0.974 a ± 0.000372	0.555 a ± 0.000455	0.216 a ± 0.0000990
Mixture 2	10.7 e ± 0.0630	35.5 bc ± 0.0251	1.15 a ± 1.90	1.82 a ± 0.0185	0.713 bc ± 0.000754	0.355 bc ± 0.000267
Mixture 3	10.2 d ± 0.138	35.1 b ± 0.0248	1.73b ± 1.26	0.992 a ± 0.000145	0.572 a ± 0.000243	0.306 ab ± 0.0000886
Mixture 4	9.44 c ± 0.0129	34.6 b ± 0.315	1.24 a ± 1.67	1.44 a ± 0.0113	0.681 b ± 0.000236	0.380 bc ± 0.000185

Table 4. Colour parameters in the CIEL*a*b* system of the different gluten-free breads as well as the wheat bread (control sample). Values are means of three determinations ± RSD and the composition of the breads is indicated in Table 1. Values followed by the same letters within each parameter indicate no significant differences in the one-way ANOVA test (p>0.05).

Bread	Crust			Crumb		
	L*	a*	b*	L*	a*	b*
Wheat	50.8 a ± 0.734	11.2 d ± 0.00638	21.7 a ± 0.180	60.8 b ± 2.52	-1.27 a ± 0.00318	9.56 b ± 0.141
Mixture 1	61.4 b ± 1.25	8.13 c ± 0.0507	27.3 b ± 0.467	51.2 a ± 1.47	2.02 c ± 0.0302	12.8 c ± 0.463
Mixture 2	74.5 c ± 2.78	2.36 b ± 0.00672	23.2 a ± 0.245	59.7 b ± 1.63	-0.518 b ± 0.000916	6.64 a ± 0.0640
Mixture 3	78.0 d ± 1.69	1.43 a ± 0.00512	21.5 a ± 0.388	59.7 b ± 1.88	-0.639 ab ± 0.00127	5.93 a ± 0.0406
Mixture 4	76.9 c ± 1.88	2.16 b ± 0.00571	21.8 a ± 0.199	59.6 b ± 2.06	-0.569 ab ± 0.00120	6.43 a ± 0.0599

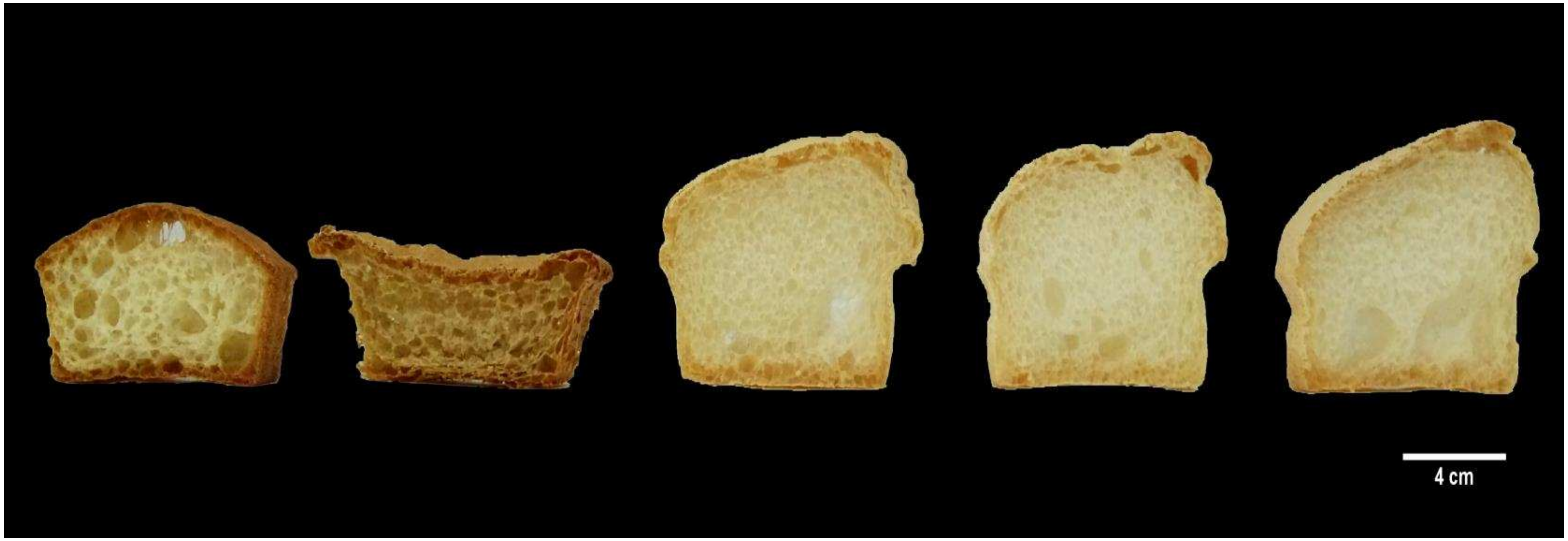

Fig.1. Crumb structure of wheat bread (control sample) and gluten-free breads made with different starch and flour mixtures. From the left to the right, wheat bread, mixture 1, mixture 2, mixture 3 and mixture 4.

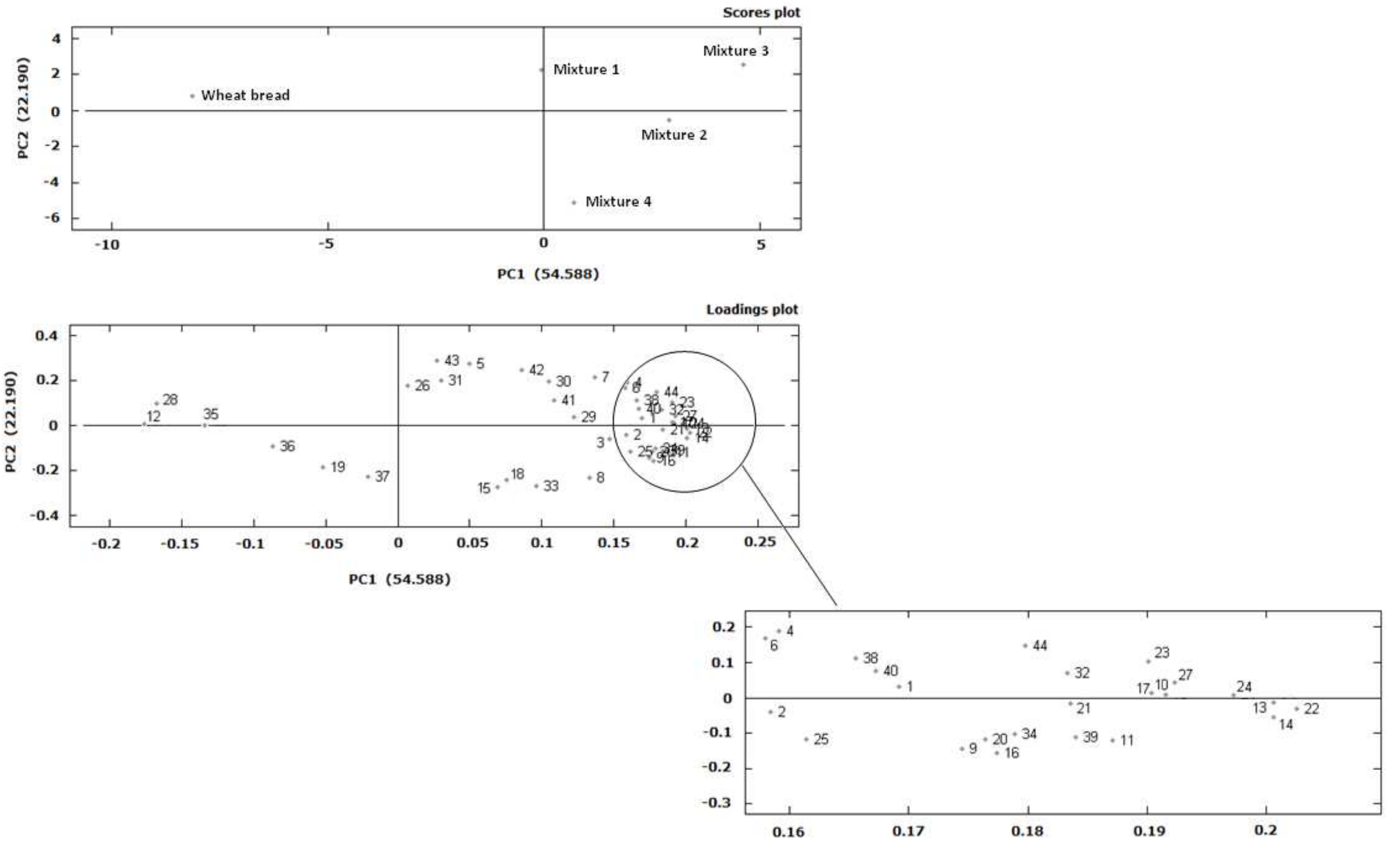

Fig.2. PCA of the four gluten-free breads as well as the wheat bread (control sample) analysed semi-quantitatively by SPME-GC/QTOF (peak areas represented). The scores plot represents the 5 samples and the loadings plot depicts the 44 volatile compounds selected. The numbers corresponding to each volatile compound are indicated in Table S1.

Table S1. Kovats index (KI), odour thresholds (OT) and organoleptics characteristics of the 58 volatile compounds studied among the four gluten-free breads as well as the wheat bread. The numbers corresponding to the labels in the PCA (Figure 1) are also indicated.

Volatile compound	Number	KI calculated	KI literature	OT[e] (µg Kg^{-1})	Organoleptic [a,b,c,d] characteristics
2,3-Butanedione SS	1	1004	984	6.5	Buttery
Hexanal ST	2	1060	1080	4.5	Green grass
2-Methyl-1-propanol SS	3	1073	1052	3200	Wine, malty
1-Methylpyrrol SS	4	1046	1140	37	Toasted
Heptanal SS	5	1170	1168	3	Fatty, pungent
R-Limonene SS	6	1185	1202	10	Citrus
Pyrazine SS	7	1205	1216	100	Nutty
2-Methyl-1-butanol ST	8	1212	1218	40000	Sweet
3-Methyl-1-butanol ST	9	1213	1218	250	Balsamic, alcohol
1-Pentanol SS	10	1254	1257	4000	Fusel-like
2-Methylpyrazine ST	11	1263	1268	105	Green, nutty, cocoa
Acetoin ST	12	1281	1286	800	Buttery
2-Octanone SS	13	1284	1283	50	Cheesy, musty
2,5-Dimethylpyrazine ST	14	1316	1316	800	Chocolate,earthy
2,6-Dimethylpyrazine ST	15	1322	1319	200	Chocolate, fries
2-Ethylpyrazine ST	16	1327	1323	6000	Musty,nutty,peanut
2,3-Dimethylpyrazine ST	17	1339	1325	2500	Green, nutty, cocoa
2-ACPY ST	18	1341	1330	0.053	Roasted, popcorn
1-Hexanol ST	19	1354	1359	2500	Sweet alcohol
Nonanal SS	20	1390	1396	1	Waxy, green, fatty
2,3,5-trimethylpyrazine ST	21	1395	1396	400	Nutty
2-Ethyl-3-methylpyrazine ST	22	1395	1400	0.4	Potato, burnt nutty
Ethyl octanoate ST	23	1433	1437	92	Fruity, floral
1-Octen-3-ol ST	24	1451	1456	1	Mushroom
Acetic acid ST	25	1453	1465	32300	Vinegar-like
Furfural ST	26	1461	1467	3000	Woody, almond

Table S1. (continued)

Volatile compound	**Number**	**KI calculated**	**KI literature**	**OT[e] (µg Kg^{-1})**	**Organoleptic [a,b,c,d] characteristics**
2-Ethyl-1-hexanol [ST]	27	1489	1489	138	Sweet, floral
Benzaldehyde [ST]	28	1510	1521	350	Bitter almond
2-(E)-Nonenal [ST]	29	1528	1546	0.08	Green,tallow
5-Methyl-2-furaldehyde [ST]	30	1565	1574	16000	Sweet, caramellic
Butyrolactone [ST]	31	1609	1622	20000	Sweet, caramel
2-Acetylpyrazine [SS]	32	1612	1614	62	Creamy
Butyric acid [ST]	33	1622	1636	240	Rancid, sweaty
Phenylacetaldehyde [ST]	34	1627	1642	4	Honey-like
Furfuryl alcohol [ST]	35	1652	1666	8	Coffee
2-Methylbutanoic acid [ST]	36	1662	1674	1600	Cheesy, rancid
3-Methylbutanoic acid [ST]	37	1662	1679	120	Rancid, sweaty
2,4-(E,E)-Decadienal [ST]	38	1797	1797	0.1	Fatty, deep-fried
Hexanoic acid [ST]	39	1900	1880	3000	Fatty
Benzyl alcohol [ST]	40	1951	1893	10000	Fruity, balsamic
Phenylethyl alcohol [ST]	41	2029	1942	1100	Rose-like
2-Acetylpyrrol [ST]	42	2164	1950	170000	Nutty, musty
4-Hydroxy-2,5-dimethyl-3(2H)-furanone [ST]	43	2203	2020	30	Caramel-like
4-Vinylguaiacol [ST]	44	2253	2230	3	Amber, cedar
3-Methylbutanal [ST]	45	989	938	0.2	Apple-like
2-Pentylfuran [ST]	46	1238	1241	6	Floral, fruit
Octanal [ST]	47	1280	1278	0.7	Strong, fruity
Ethyl heptanoate [ST]	48	1329	1328	2.2	Pineapple, fruity
2-Ethyl-5-methylpyrazine [ST]	49	1377	1377	100	Nutty, roasted
3-Furaldehyde [ST]	50	1455	1458	23000	Almond
Ethyl nonanoate [ST]	51	1538	1538	850	Fruity, rose
Ethyl decanoate [ST]	52	1628	1624	510	Grape, fruity

[a] https://pubchem.ncbi.nlm.nih.gov/compound/

[b] http://www.pherobase.com

[c] http://www.thegoodscentscompany.com

[d] Birch, Petersen & Hansen (2013).

[e] http://www.leffingwell.com/odorthre.htm

SS = Splitless; ST = Split

X. Discussion of the results

Discussion of the results

The present Doctoral Thesis approaches the study of volatile compounds in gluten-free breads with the aim of developing a final recipe for an improved aroma. For this purpose, it was necessary to achieve specific goals that started with the development of solvent extraction methodologies as well as new strategies for obtaining accurate results. With the developed solvent extraction methodologies and the optimised headspace methodologies, the volatile profiles of different gluten-free flours, starches, doughs, crumbs and crusts were determined and/or quantified, ascertaining the most suitable mixture of gluten-free flours and starches that gave rise to a stronger pleasant gluten-free bread aroma.

Through **Section 1**, the necessity of examining solvent extraction methodologies was elucidated, showing that only with the most suitable methodology would it be possible to obtain good results. Solvent extraction methodologies and headspace extractions have been the two options in the analysis of volatile compounds, thus both alternatives were used for this Doctoral Thesis. Solvent extraction methodologies were studied first because they were the most tedious and a good alternative for the crumb, which contained little amount of fat that hindered the chromatographic analysis (column clogging, dirtiness in the injector, interferences in the chromatogram…). On the other hand, headspace methodologies were a better choice for crust aroma analyses due to the limited amount of crust available in gluten-free breads. Up to now, SAFE has been the most commonly applied solvent extraction methodology, but it requires a fragile glass device that is very tricky to clean. Additionally, when the validation of the method for the analysis of bread crumb aroma was tried, we found low extraction efficiencies (average 52 %) and unsuitable % RSD for the intermediate precision (average of 15 %). Therefore, an alternative solvent extraction methodology using lipases (LM) was proposed, which consisted of an extraction with a mixture diethyl ether/ dichloromethane (2:1) that contained lipases, which effectively hydrolysed the fat into free fatty acids and glycerol. These compounds eluted in the final part of the chromatogram without causing interferences with the volatile compounds, obtaining cleaner extracts that helped preserve the life of the GC. The volatile profile obtained was completely analogous to that of commercial wheat breads reported in the literature, proving the validity of the LM. Furthermore, since the distillation step was not performed in LM, there was an improvement on the extraction efficiencies (average of 87% for LM) and % RSD for intermediate precision (average of 5 % for LM). However, when LM was applied to gluten-free breads, there were problems in reducing the volume of the extract when the fat content was higher than 2% (i.e. teff, buckwheat and quinoa). As a consequence, when the fat content was higher than 2%, it was necessary to utilise the SM, due to the physical removal of the non-volatile compounds.

In order to become acquainted with the available literature regarding wheat bread aroma, the work in Section 1 was developed with a commercial wheat bread that was freshly analysed. However, the results of gluten-free breads were not predictable due to the novelty of the work, especially with the necessity of freezing. Moreover, when the dough was waiting to be analysed, residual fermentation was observed, and the septum of SHS usually exploded during the first minutes of analysis as a consequence of the carbon dioxide produced in this residual fermentation. Overdone freezing time in the case of the crumb or evolution of the residual fermentation in the case of the dough, would lead to inaccurate results. In **Section 2**, consequently, strategies for the freezing of bread crumb and the inhibition of fermentation evolution in prepared doughs were developed. It was determined that a maximum of one week of freezing was advisable for solvent extraction aroma analyses, in order to avoid average losses of volatile compounds higher than 24 % or changes in the volatile profile due to chemical reaction after two weeks. In the case of headspace aroma analyses, analysis on the same day as bread preparation was recommended, since the acceleration of the retrogradation of starch during freezing facilitates the release of volatile compounds from the matrix, leading to erroneous results. In the case of the inhibition of fermentation evolution in doughs, a mixture of methyl octanoate and methyl decanoate (Fames) was suggested as a non-toxic alternative to $HgCl_2$. The use of the Fames mixture allowed an efficient termination of the fermentation evolution in few minutes prior to aroma analyses, without interferences in the chromatogram. Thus, there was an increase in the concentration of each volatile compound with the increase in the fermentation time, a logical progression that was lost when Fames were not added.

With the tools for the analysis of bread aroma available, it was possible to conduct gluten-free bread aroma analyses of flours and starches, doughs, crumbs and crusts. **Section 3** was dedicated to the analysis of volatile compounds in gluten-free doughs and crumbs as well as its evolution from different fermentation times for the baked crumb. From the evolution of the volatile compounds from dough to crumb it was concluded that the dough, at different fermentation times, was characterised by pleasant volatile compounds from fermentation, such as 3-methyl-1-butanol, 2,3-butanedione, acetoin, butyric acid and ethyl octanoate. In the crumb, alongside dough compounds, hexanal, 1-octen-3-ol and nonanal from lipids oxidation were also important contributors. The evolution was characterised mainly by volatile compounds that increase during fermentation and can be evaporated during baking (i.e. 3-methyl-1-butanol, 1-propanol or 2,3-butanedione) or increased during baking (acetoin, phenylethyl alcohol, nonenal or 2,4-decadienal); only the redox pairs hexanal-hexanoic acid, benzaldehyde-benzyl alcohol and furfural- furfuryl alcohol remained steady during fermentation. The verification of the evolution of the volatile compounds led to the idea that different gluten-free flours and starches should provide different volatile profiles in dough and crumb due to the presence of different precursors. A screening with SHS-GC/MS of different gluten-free doughs and crumbs elaborated with yellow and white corn, rice, oat, teff, buckwheat, amaranth and quinoa flours and wheat, corn and potato starches, confirmed this evolution. Corn starch, quinoa, amaranth, buckwheat, rice and teff bread crumbs were selected for study by DHS-GC/MS and the same conclusions as those obtained with the PCA of the crumbs analysed by SHS-GC/MS were extracted: (i) There was a clear separation between gluten-free cereals and pseudocereals; (ii) Quinoa flour and corn starch were selected for the improvement of the aroma of gluten-free bread crumb. Quinoa crumb showed a high content of pleasant alcohols from fermentation (as a consequence of its high content in α-glucosidase), such as 3/2-methyl-1-butanol, and a low content of off-flavours from lipids oxidation, like hexanal and 2,4-decadienal (as a consequence of the high content in antioxidants and the low lipoxygenase activity). Corn starch crumb was selected for its high content of 3-methyl-1-butanol (SHS) and 2,3-butanedione and furfural (DHS); (iii) It was also concluded that rice crumb was excluded due to its high content of fatty-rancid volatiles from lipids oxidation, mainly nonanal and 2,4-decadienal (as a consequence of its high lipoxygenase activity and its low antioxidant content). Teff crumb was the only sample that differed between SHS and DHS analyses, since in DHS it was characterised by high contents in alcohols (1-octen-3-ol, 1-pentanol, 1-hexanol) and aldehydes (pentanal, hexanal, heptanal) from lipids oxidation, while in SHS it presented higher levels of alcohols from fermentation. However, in both the PCA of the SHS and DHS analyses, teff was located in the same PC1 as the pseudocereals and in the opposite PC1 as the gluten-free cereals. Finally, both SHS and DHS analyses indicated that all the gluten-free bread crumbs and wheat bread crumb presented the same volatile compounds but in different concentration.

As there were differences in the proportion of volatile compounds among the different gluten-free bread crumbs, **Section 4** was devoted to the analysis of volatile compounds in flours and starches as well as in gluten-free bread crusts. The aim of analysing the volatile compounds of flours and starches was to check if the volatile compounds were in different proportion in crumb because they came from the flour itself or because they were generated from the flours and starches' precursors. As the volatile compounds in flours and starches should be present in traces, SAFE-GC/QTOF, SHS-GC/QTOF and SPME-GC/QTOF methodologies were developed, optimised and analytically compared with the intention of using the most suitable methodology. Due to the large number of volatile compounds detected, the low LODs, the good repeatability and the quickness and simplicity of the sample treatment, SPME-GC/QTOF methodology was selected as the best option. The PCA of the volatile compounds from gluten-free flours (quinoa, buckwheat, rice and teff) and starches (corn) showed a clear separation of quinoa flour and corn starch, coincidentally the two flour bases that were suggested for the improvement of the aroma of the gluten-free bread crumb. However, the volatile profiles presented by the flours and starches were completely different from the corresponding crumbs: (i) Quinoa flour and corn starch were highlighted for their content in pyrazines, terpenes, benzenic compounds and esters, while the corresponding crumbs stood out for the alcohols from fermentation (e.g. 3-methyl-1-butanol), acetoin, 2,3-butanedione Ehrlich aldehydes and acetic acid ; (ii) Teff flour presented high contents of 3-methyl-1-butanol, acetoin, 4-vynilguaiacol and organic acids, while terpenes, benzenic compounds, esters and alcohols and aldehydes from lipids oxidation were the main volatile compounds in the corresponding crumb. Most of the volatile compounds that were important in quinoa crumb and corn starch crumb were relevant in teff flour and vice versa. Therefore, gluten-free flours and starches did not supply volatile compounds and mostly provided the precursors of these volatile compounds. Given the results of the volatile profiles in gluten-free flours/starches and gluten-free bread crumbs, it was thought that the main differences between gluten-free breads should be due to the crust. For this purpose, SPME-GC/QTOF methodologies in semi-quantitative (splitless) and quantitative (split 1:100) modes were developed, optimised and validated. Semi-quantitative aroma analyses of basmati rice, japonica rice, oat, teff, quinoa, corn starch and wheat starch bread crusts were made in order to select the most suitable crusts for quantification. The crusts were mainly distinguished by their content in pyrazines, 2-acetyl-1-pyrroline, 2-(E)-nonenal and 2,4-(E,E)-decadienal, with japonica rice and teff bread crusts exhibiting volatile profile that was most similar to wheat bread crust (control sample). Wheat starch crust was also selected for its high content in pyrazines and basmati rice crust for trying the effect of the variety of rice. From the quantification it was concluded that basmati rice and wheat flour crusts were distinguished from the rest in the PC1 due to the high content in 2-(E)-nonenal. The separation in the PC1 of japonica rice, wheat starch and teff crusts was due to the high content in pyrazines, although japonica rice crust was slightly separated from the rest due to its high content in 1-octen-3-ol, heptanal and 2,4-(E,E)-decadienal. Japonica rice crumb was also characterised by off-flavours from lipids oxidation, while teff and wheat starch crumbs were not characterised by pyrazines. Additionally, teff crust was characterised by its high contents of pleasant fermentation compounds, like acetoin, phenylacetaldehyde or 3-methylbutanoic, contrary to teff crumb (characterised by lipid oxidation volatile compounds). Thus, it was suggested that the transference of fermentation volatile compounds from crumb to crust was easier in the case of teff bread and that the generation of volatile compounds from lipids oxidation was more intense in japonica rice crust than in the other crusts. Finally, wheat bread crust was characterised by the highest content in 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 2-acetyl-1-pyrroline, which were really similar in teff and wheat starch crusts. Therefore, a mixture of teff flour and wheat starch was suggested for the improvement of gluten-free bread crust. As in the case of the gluten-free crumbs, most of the volatile compounds were the same between the examined gluten-free bread crusts but in different concentrations. Therefore, the human perception of different gluten-free breads should be based on the proportion between the volatile compounds but not on specific volatile compounds.

Taking into consideration the conclusions extracted from Section 3 and 4, **Section 5** aimed to find the suitable proportion between quinoa flour and corn starch (improvers of the crumb aroma) and teff flour and wheat starch (improvers of the crust aroma) for the enhancement of the final aroma of gluten-free bread. The use of quinoa flour was limited for its content in saponins, the content of teff flour was limited for the volatile compounds from lipids oxidation that were generated in its crumb and the use of starches was limited given their excessively light and crumbly crust. Due to the better physical properties, the higher amounts of pleasant volatile compounds (such as 2-acetyl-1-pyrroline and 3-methyl-1-butanol) and the lower amount of off-flavours from lipids oxidation, the recipe with 40 % of wheat starch, 40 % of corn starch, 15 % of quinoa flour and 5 % of teff flour was suggested as the most suitable for the improvement of the aroma of gluten-free bread. It was also concluded that the powerful flavour that has been usually reported for wheat bread should be related to the proportion between the different compounds, since wheat bread presented the highest levels only in 12 of the 52 detected volatile compounds. Therefore, the differences between gluten-free breads and wheat bread should be based on the concentration of the most important volatile compounds and not on specific volatile compounds that are present or absent in their volatile profiles.

XI. Conclusions

Conclusions

The main objective of this Doctoral Thesis has been to improve the aroma of gluten-free breads through the examination of the influence of different gluten-free flours and starches on the volatile profile of the corresponding doughs, crumbs and crusts. It was necessary to develop and validate different analytical methods, including GC/MS solvent extraction and headspace methodologies, as well as strategies to ensure the most accurate results as possible. It was finally concluded that a mixture of 40 % corn starch, 40 % wheat starch, 15 % quinoa flour and 5 % teff flour was a suitable mixture for the improvement of the final aroma of gluten-free bread analysed by SPME-GC/QTOF, since it presented high proportion of pleasant volatile compounds for fermentation, pyrazines, 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 2-acetyl-1-pyrroline and low proportion of off-flavours from lipids oxidation. Moreover it showed good physical properties, such as colour, volume and texture. For the achievement of this general aim, specific conclusions were reached:

1- The developed and validated lipases solvent extraction methodology was proven to greatly improve the extraction efficiencies and % RSD of the intermediate precision of the SAFE methodology in the analysis of bread crumb aroma.

2- A maximum of one week of frozen storage was recommended for solvent extraction analyses of bread crumb, while the determination in the same day of bread preparation was advised for headspace analyses of bread crumb.

3- A mixture of methyl octanoate and methyl decanoate acted as an efficient inhibitor of fermentation evolution in bread doughs for aroma analyses, avoiding the toxicity of the classical $HgCl_2$.

4- Examination of the evolution of volatile compounds from dough to crumb in gluten-free bread showed that the dough was characterised by volatile compounds from fermentation and the crumb by the dough volatile compounds as well as volatiles from lipids oxidation.

5- SHS-GC/MS and DHS-GC/MS analyses of different gluten-free bread crumbs led to the same conclusion: quinoa flour and corn starch were suggested as the best option for the improvement of gluten-free bread crumb aroma due to the high content of pleasant volatile compounds from fermentation, like 3-methyl-1-butanol, and the low content of off-flavour lipids oxidation volatile compounds, like 2,4-(E,E)-decadienal.

6- SPME-GC/QTOF quantitative analyses of gluten-free flours and corn starch led to the conclusion that the flour bases simply provide the precursors for the generation of volatile compounds.

7- SPME-GC/QTOF quantitative analyses of different gluten-free bread crusts showed that teff flour and wheat starch were the best options for the improvement of gluten-free bread crust aroma due to their similar content of 2-acetyl-1-pyrroline and 4-hydroxy-2,5-dimethyl-3(2H)-furanone compared to wheat bread, as well as the high content of pyrazines of wheat starch crust and pleasant volatile compounds from fermentation of teff crust.

Annex

Table 1A. Important aroma compounds reported in wheat bread with OAV > 0.1 and FD > 8. The OT in water (µg/kg) is also specified

Volatile Compound	OAV	FD Factor	OT
2-(E)-Nonenal	40–65[d]	256 and 128[b], 512[a]	0.08
3-Methylbutanal	25–54[d] and 56–236[c]	…	0.2
Nonanal	15–20[d] and 18–34[c]	…	1
Hexanal	12–20[d] and 49–87[c]	16[a]	4.5
Phenylacetaldehyde	8–13[d] and 6–35[c]	<8 and 16[b]	4
Heptanal	5–13[d] and 14–26[c]	…	3
Octanal	4–7[d] and 19–30[c]	…	0.7
Decanal	2–4[d] and 8–21[c]	…	2
Benzaldehyde	0–0.2[d] and 0.1–0.2[c]	…	350
2,4-(E,E)-Decadienal	…	512[a]	0.07
Trans-4,5-Epoxy-(*E*)-2-decenal	…	512[a]	0.02
2-(Z)-Nonenal	…	128[a]	0.02
Methional	…	16 and 64[b], 64 and 128[a]	0.04
2,4-(E,Z)-Decadienal	…	64[a]	0.1
2,4-(E,E)-Nonadienal	…	32 and 128[a]	0.06
4-(Z)-Heptenal	…	16[a]	0.06
2-(E)-Octenal	…	16[a]	3

Table 1A. (continued)

Volatile Compound	OAV	FD Factor	OT
3-Methyl-1-butanol	17–25[d] and 25–35[c], 18 and 10[b]	128 and 32[b], 32 and 256[a]	250
1-Octen-3-ol	9–13[d]	…	1
1-Heptanol	2–3[d] and 8–14[c]	…	3
2-Methyl-1-propanol	0.1–0.3[d] and 0.2–0.8[c]	…	3,2
2-Phenylethanol	0.2–0.4[d] and 0–0.1[c], 12 and 3[b]	512 and 128[b], 4 and 256[a]	1,1
1-Propanol	0.1–2[e]	…	6,6
2,3-Butanedione	9–33[d] and 19–103[c]	8 and 16[b], 64 and 128[a]	6.5
6-Methyl-5-hepten-2-one	2–5[c]	…	50
3-Hydroxy-2-butanone	0.8–1.5[c]	…	800
1-Octen-3-one	…	<8 and 64[b], 128 and 128[a]	0.01
2-Octanone	0.1–0.2[d]	…	50
1,5-(Z)-Octadien-3-one	…	16[a]	0.0004
3-Methylbutanoic acid	5.5 and 15[b]	<8 and 32[b]	120
Acetic acid	…	64 and 128[b]	32,3
Butanoic acid	<1[b]	<8 and 32[d]	240
Ethyl 3-methylbutanoate	1.5–11[d]	…	0.2
Ethyl hexanoate	0.2–0.5[d]	…	1

Table 1A. (continued)

Ethyl octanoate	0–0.1^{c}, 19 and 15^{b}	512 and 128^{b}	92
Volatile Compound	**OAV**	**FD Factor**	**OT**
Ethyl-2-methyl propanoate	…	16^{c}	4.5
Ethyl acetate	0–0.1^{c}	…	6,2
Vanillin	…	<8 and 16^{b}	22
2-Pentylfuran	1–3^{c,d}		6
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	…	8 and 64^{b}	40
4-Vinyl-2-methoxyphenol	…	16 and 64^{b}	3
2-Acetyl-1-pyrroline	…	8b and 32^{a}	0.053

a = Schieberle and Grosch (1991); when two FD factors are listed, they represent wheat dough fermented for a short time and a long time. b = Gassenmeier and Schieberle (1995); the two FD factors represent dough fermentation by 15 and 46 g of yeast/kg of flour. c = Birch et al (2013a);. d = Birch et al (2013b)

Table 2A. Volatile alcohols found in wheat bread (crumb and/or crust) reported in literature their typical odours. Volatile compounds are in descending order by times cited.

Alcohols	Crumb / Crust	Odour	References [c]
3-Methyl-1-butanol	Crumb, crust	Balsamic, alcoholic, malty	1,2,5,6,7,8,11,12,14,16,17,19,21,22,26,29,30,31, 32,33,34,36,38,42,43,47,49,50
Ethanol	Crumb, crust	Alcoholic	2,6,7,8,11,12,14,16,17,19,22,26,29,30,31,32,33,34,42,44,49
2-Methyl-1-propanol (isobutyl alcohol)	Crumb, crust	Glue, alcoholic, wine-like, malty	2,1,5,6,7,8,11,12,14,16,17,19,26,30,31,32,33,34, 38,42
1-Hexanol	Crumb, crust	Green grass, flowery, woody, mild, sweet	2,1,5,6,8,11,12,14,16,22,26,30,31,32,33,34, 42,47,49
1-Pentanol	Crumb	Balsamic, fruity, fusel-like sweet	2,1,5,6,7,11,12,16,17,26,30,32,34,42,49
1-Propanol	Crumb	Fruity, alcoholic, plastic,pungent	2,1,5,6,7,8,11,16,17,19,31,32,34,42,49
2-Phenylethanol	Crumb, crust	Flowery, yeast-like, honey	1,5,9,12,16,30,32,33,36,41,42,43,50
2-Methyl-1-butanol	Crumb, crust	Malty	2,6,7,11,16,26,31,32,34,42,43,50
1-Butanol	Crumb	Fruity, solvent	2,1,5,6,12,16,17,32,42
1-Octanol	Crumb, crust	Earthy, mouldy vegetable	2,1,5,6,12,14,26,30,34
1-Heptanol	Crumb	Green	2,1,5,6,12,26,30,34,50
Benzyl alcohol	Crumb	Pleasant aromatic	12,14,16,23,26,30,33,42,44
1-Octen-3-ol	Crumb, crust	Mushroom-like	5,7,11,12,26,30,32
3-Nonen-1-ol	Crumb	Waxy	5,14,23,26,30
2-Methoxy-4-vinylphenol (4-Vinylguaiacol)	Crumb	Spicy	10,23,26,41,45
Phenylethyl alcohol	Crumb, crust	Rose-honey-like, wilted rose	7,8,23,26
Phenylethanol	Crumb, crust	Flowery	14,26,44
2-Ethyl-1-hexanol	Crumb, crust	Green, vegetable	1,5,26
2,3-Butanediol	[b]Bread	Neutral smelling	7,16,32
1-Nonanol	Crumb, crust	Citrus	26,30,38
Phenol	Crumb, crust	Sweet and tarry	26,30,38
1-Penten-3-ol	Crumb	Burnt, butter, grass,	5,32,50

Table 2A. (continued)

Alcohols	Crumb / Crust	Odour	References [c]
2-Octen-1-ol	Crumb	Green, vegetable-like	1,26
2-Penten-1-ol	Crumb	Green type	1,5
3-Methyl-3-buten-1-ol	Crumb	Fruity, green	1,5
2-Butanol	Crumb	Alcoholic	2,6
2-Hexanol	Crumb	Winey type	2,6
2-Hexenol	Crumb	Green type	2,6
Decanol	Crumb, crust	Fatty type	23, 38
3-Pentanol	Crumb	Herbal type	16
2-Ethyl-1-decanol	[b]Bread	Citrus type	14,44
3-(Methylthio)-1-propanol	Crumb (traces)	Potato	43
2-Ethyl-1-ethanol	Crumb, crust	Green, vegetable	30
Guaiacol	Crumb, crust	Soapy, sweet, burnt	45
3-Ethoxy-1-propanol	Crumb, crust	Fruit	11
Geosmin	[b]Bread	Musty	39
4-Decen-1-ol	Crumb	Green odour	26
5-Methyl-2-furanmethanol	Crust	Honey, sweet	26
3-Decen-1-ol	Crumb	[a]nf	30
2-Propanol	Crumb	Pungent smell	16
2-Pentanol	Crumb	Fermented type	16
3-Hexenol	Crumb	Grassy-green	29,5
2-Cyclohexenol	[b]Bread	[a]nf	23
2-Heptanol	[b]Bread	Citrus type	12
4-Methyl-4-nonenol	[b]Bread	[a]nf	12
2-Nonen-1-ol	[b]Bread	Waxy melon	14
1-Methoxy-2-propanol	[b]Bread	Mild ether odour	32
2-Undecanol	[b]Bread	Fruity type	14
2,4-Bis(1,1-dimethylethyl)phenol	[b]Bread	Weak aromatic	23
1-Dodecanol	Crumb	Waxy type	5
Nitrobenzol	Crumb, crust	Bitter almond	38

[a]nf = not found in literature.

[b]Bread = crumb and crust were analysed together (mixed) in the mentioned article. Therefore, there is no knowledge if the volatile compound came from the crumb or crust.

[c]1. (Birch et al., 2013)
2. (Hansen & Schieberle, 2005)
3. (Czerny & Schieberle, 2002)
5. (Birch et al., 2013)

6. (Hansen & Hansen, 1994)
7. (Poinot et al., 2008)
8. (Poinot et al., 2010)
9. (Moskowitz et al., 2012)
10. (Gassenmeier & Schieberle, 1995)
11. (Jensen et al., 2011)
12. (Plessas et al., 2011)
13. (Cho & Peterson, 2010)
14. (Plessas, Bekatorou et al., 2008)
16. (Martínez-Anaya, 1996)
17. (Torner et al., 1992)
19. (Martínez-Anaya et al., 1990)
21. (Schieberle & Grosch, 1991)
22. (Schieberle & Grosch, 1985)
23. (Lin, Hsieh, Liu, Lee, & Mau, 2009)
26. (Paraskevopoulou et al., 2012)
29. (Bianchi et al., 2008)
30. (Ruiz, Quílez, Mestres, & Guasch, 2003)
31. (Seitz et al., 1998)
32. (Luning et al., 1991)
33. (Quílez et al., 2006)
34. (Uhr-Rehman et al., 2006)
36. (Grosch & Schieberle, 1997)
37. (Schieberle & Grosch, 1987a)
38. (Obretenov & Hadjieva, 1977)
39. (Keshri et al., 2002)
41. (Zehentbauer & Grosch, 1998a)
42. (Hansen & Hansen, 1996)
43. (Onishi et al., 2011b)
44. (Plessas, Fisher et al., 2008)
45. (Rychlik & Grosch, 1996)
47. (Schieberle & Grosch, 1987b)
49. (Poinot et al., 2007)
50. (Frasse et al., 1993)

Table 3A. Volatile aldehydes found in wheat bread (crumb and/or crust) reported in literature their typical odours. Volatile compounds are in descending order by times cited.

Aldehydes	Crumb / Crust	Odour	References [c]
Hexanal	Crumb, crust	Green, grassy, tallow	1,2,3,5,6,7,11,12,14,16,17,19,21,22,26,30,32,33,34,41,44,45,47,49,50
3-Methylbutanal	Crumb, crust	Malty, roasty cucumber-like	1,3,5,7,11,12,13,14,16,22,26,29,31,32,36,41,42,45,47,49,50
2-(E)-Nonenal, 2-(Z)-Nonenal	Crust (Z), Crumb & crust (E)	(E): fatty, green, tallowy, paper, cucumber-like (Z): fatty, tallowy, green	3,5,9,10,12,13,14,16,21,22,26,29,30,31,33,36,41,43,45,47,50
Benzaldehyde	Crumb, crust	Almond, caramel	1,2,5,7,8,11,12,14,16,23,26,30,31,32,33,34,38,42,47
2,4-(E,E)-Decadienal, 2,4-(E,Z)-Decadienal	Crumb, crust	(E,E): deep fat fried, waxy (E,Z): deep fat fried	3,9,12,13,16,21,23,26,30,33,36,41,45,47,50
Phenylacetaldehyde	Crumb, Crust (traces)	Honey-like, sweet	1,3,5,10,13,16,22,26,36,41,43,45,47
Nonanal	Crumb, crust	Citrus, soapy	1,5,11,12,16,22,23,26,30,32,34,41,47
2-Methylbutanal	Crumb, crust	Almond, malty	1,7,11,12,16,26,29,32,34,41,45,49
Heptanal	Crumb, crust	Fatty, rancid, citrus, malty	1,5,11,12,14,16,26,32,34
2-(E)-Heptenal	Crumb, crust	Green, fatty	2,6,16,22,26,30,31,32,47
3-(Methylthio)-propanal (methional)	Crumb, crust	Boiled-potato, cooked-potato, malty, waxy	1,9,10,13,21,36,41,43,50
Acetaldehyde	Crumb, crust	Fruity	12,14,16,17,19,26,34,45
4-(Z)-Heptenal	Crust	Biscuit-like, sweet, putrid	1,13,16,21,22,41,45,47
2-(E)-Octenal	Crumb, crust	Fatty, nutty, roasted	16,21,22,26,30,31,45,47
5-Methyl-2-furfural	Crust	Almond, sweet, bitter	7,8,16, 22, 26,32, 47, 49
2-Methylpropanal	Crumb, crust	Malty	7,9,16,26,32,41,45,49
Octanal	Crumb, crust	Citrus, flowery	1,5,16,26,30,32,34
2,4-(E,E)-Nonadienal, 2,4-(E,Z)-Nonadienal	Crumb, crust	(E,E), (E,Z): deep fat fried	3,13,21,26,41,45
2,6-(E,Z)-Nonadienal	Crumb, crust	Cucumber-like	3,13,16,22,41,47
Vainillin	Crumb, crust	Vanilla-like	1,10,26,41,43,45
4,5-Epoxy-2-decenal	Crumb, crust	Metallic	10,21,41,43,45
Decanal	Crumb	Citrus	1,5,26,29,32

Table 3A. (continued)

Aldehydes	**Crumb / Crust**	**Odour**	**References** [c]
Pentanal	Crumb (traces)	Strong acrid, pungent	16,26,30,32
2-Hexenal	Crumb, crust	Green, fatty	16,26,47
3-Furfural	Crumb (traces)	Almond-like	7, 8, 16
2-Butenal	Crust	Pungent, suffocating	16,26
2-Methyl-2-butenal	Crust	Green type	26,29
2-Decenal	Crust	Metallic	26
Butanal	Crumb	Malty	16
Propanal	Crumb	Malty	16
2-Undecenal	Crust	Fruity type	26
2-Methyl-2-propenal	Crumb	[a]nf	29
Formaldehyde	Crumb	Pungent	16
2-Oxopropanal	Crumb	Pungent stinging	16
2-Propenal	Crumb	Cooking grease	16
2-Phenyl-propenal	Crumb	Cinnamon	16
2-Methylpentanal	Crumb	Sweet fruity green	16
2-Ethylhexanal	Crumb	Powerful, strong	16
2-Iso-octenal	[b]Bread	Green	38
Dodecanal	[b]Bread	Aldehydic type	38
2-Hydroxybenzaldehyde	[b]Bread	Medicinal	23
4-Hydroxybenzaldehyde	Crumb	Medicinal	16
Phenylethanal	[b]Bread	[a]Floral-green	23
Hexadecanal	[b]Bread	Burnt plastic, cardboard	14

[a]nf = not found in literature.

[b]Bread = crumb and crust were analysed together (mixed) in the mentioned article. Therefore, there is no knowledge if the volatile compound came from the crumb or crust.

[c] The numeration corresponds with the footnotes of Table 2A.

Table 4A. Volatile ketones found in wheat bread (crumb and/or crust) reported in literature their typical odours. Volatile compounds are in descending order by times cited.

Ketones	Crumb / Crust	Odour	References [c]
2,3-Butanedione (diacetyl)	Crumb (traces), crust	Buttery, caramel	1,2,5,6,7,8,9,11,13,16,17,26,29,30,32,33,34, 36,41,42,43,45,47,49,50
3-Hydroxy-2-butanone (acetoin)	Crumb	Butterscotch, butter, yogurth, cream	1,2,5,6,7,8,11,16,30,33,32,49
1-Octen-3-one	Crumb, crust	Fatty, mushroom-like	10,13,16,21,22,30,41,45
2-Heptanone	Crumb, crust	Soapy, fruity, cinnamon	1,5,16,26,29,32,47,49
2-Octanone	Crumb, crust	Soapy, fruity	5,16,22,26,29,30,47
2,3-Pentanedione	Crust	Buttery	16,26,29,41,45,49
2-Butanone	Crumb, crust	Sharp, sweet	7,11,16,31,32
1-Hydroxy-2-propanone	Crumb	Pungent sweet caramel, ether	7,8,11,32
6-Methyl-5-hepten-2-one	Crumb	Herbaceous, green	1,26,30
γ-Decalactone	Crust	Sweet, soapy	26,45
3-Penten-2-one	Crust	[a]nf	16,26,32
1,5-Octadien-3-one	Crust	Green, geranium-like	13,21,45
Acetone	Crumb, crust	Ether, grape	17,19,29
3-Octen-2-one	Crumb	Earthy type	5,26,29
2-Hexanone	[b]Bread	Sharp	16,38
2-Pentanone	[b]Bread	Fruity type	16,32
3-Hexanone	[b]Bread	Fruity type	16
2,3-hexanedione	Crust	Buttery, cheesy, sweet, creamy	29
1-Hexen-3-one	Crust	Green	41
Benzophenone	Crust	Sweet aromatic slightly-rose fruity	26
2-(5H)-furanone	Crumb (traces), crust (traces)	[a]nf	26
Geranyl acetone	Crumb, crust	Floral type	26
3,4-Heptanedione	[b]Bread	[a]nf	16
2-Nonanone	[b]Bread	Fruity	38
2-Propanone	[b]Bread	Sweetish aromatic	16
2-Octen-4-one	[b]Bread	Yeasty type	38

Table 4A. (continued)

Ketones	Crumb / Crust	Odour	References [c]
2-Nonen-4-one	[b]Bread	Fruity type	38
2-Decen-4-one	[b]Bread	[a]nf	38
2-Decen-5-one	[b]Bread	[a]nf	38
1-Dodecen-3-one	[b]Bread	[a]nf	38
2-Dodecen-3-one	[b]Bread	[a]nf	38
2-Dodecen-5-one	[b]Bread	[a]nf	38
Pentadecan-2-one	[b]Bread	Spicy, Herbaceous	38
Heptadecan-2-one	[b]Bread	[a]nf	38
1-(2-furyl)-2-propanone	[b]Bread	Green type	16
1-(2-furyl)-1,2-propanodione	[b]Bread	[a]nf	16

[a]nf = not found in literature.

[b]Bread = crumb and crust were analysed together (mixed) in the mentioned article. Therefore, there is no knowledge if the volatile compound came from the crumb or crust.

[c] The numeration corresponds with the footnotes of Table 2A.

Table 5A. Volatile esters found in wheat bread (crumb and/or crust) reported in literature their typical odours. Volatile compounds are in descending order by times cited.

Esters	Crumb / Crust	Odour	References [c]
Ethyl acetate	Crumb, crust	Sweet, fruity, pineapple	1,5,6,7,8,11,12,14,16,17,23,26,29,32,34,38,42,44
Ethyl octanoate	Crumb, crust	Sweet, soap, fresh, fruity	1,2,5,6,10,11,26,30,36,45
Ethyl hexanoate	Crumb, crust (traces)	Applepeel, fruity	2,5,6,11,12,26,42
Butyl acetate	Crumb	Fruity	2,6,12,14,30,32
Ethyl lactate	Crumb	Fruity	2,6,11,30,31
Isoamyl acetate (Isopentyl acetate; 3-methylbutyl acetate)	Crumb, crust	Banana-like	17,26,32
Ethyl propanoate	Crumb	Fruity	2,6,11
Isobutyl acetate (2-methylpropyl acetate)	Crumb	Fruity, floral	12,14,30,32
Phenylethyl acetate	Crumb	Rose	5,12,26,30

Table 5A. (continued)

Esters	Crumb / Crust	Odour	References [c]
Hexyl acetate	Crumb	Fruity, spicy, herbal	3,12,26
Ethyl nonanoate	Crumb (trace)	Fruity	10,26
Methyl caproate	Crust	Fruity	16, 22
Ethyl formate	[b]Bread	Rum	16,23
Ethyl laurate	[b]Bread	Light fruity-floral	23,32
Pentyl acetate	Crumb	Fresh banana	2,6
Ethyl pentadecanoate	[b]Bread	Sweet	12,14
2-Methylbutyl acetate	Crumb	Fruity	2,6
3-Methylbutyl acetate	Crumb	Fruity	5,6
Furfuryl formate	Crumb	Ethereal	16,29
Ethyl 2-methylpropanoate	Crumb	Fruity	21
Ethyl caprate	Crust	Fruity	23
Ethyl 3-methylbutanoate	Crumb	Fruity, apple	5
Ethyl decanoate	Crumb (traces)	Fruity	26
Furfuryl acetate	Crumb, crust	Banana-like	26
Ethyl pyruvate	[b]Bread	Ethereal	16
Ethyl levulinate	[b]Bread	Fruity, green, waxy	16
Ethyl palmitate	[b]Bread	Waxy	23
Butyl formate	[b]Bread	Fruity	38
Gexyl acetate	Crumb	[a]nf	2
Pentyl formate	[b]Bread	Banana-like	32
Octyl acetate	[b]Bread	Fruity	38
Ethyl heptanoate	[b]Bread	Grapes	12
Ethyl pentanoate	[b]Bread	Fruity, orange, grass, green	12
Ethyl butyrate	[b]Bread	Fruity	32
3-hydroxybutyl acetate	[b]Bread	[a]nf	14
Acetonyl formate	[b]Bread	[a]nf	16
Ethyl benzoate	[b]Bread	Sweet, wintergreen, medicinal	38

[a]nf = not found in literature.

[b]Bread = crumb and crust were analysed together (mixed) in the mentioned article. Therefore, there is no knowledge if the volatile compound came from the crumb or crust.

[c] The numeration corresponds with the footnotes of Table 2A.

Table 6A. Volatile acids found in wheat bread (crumb and/or crust) reported in literature their typical odours. Volatile compounds are in descending order by times cited.

Carboxilic acids	**Crumb / Crust**	**Odour**	**References** [c]
Acetic acid	Crumb, crust	Sour, acid, pungent	3,7,8,10,11,12,14,16,23,26,30,31,32,33,34, 36,41,42,43,44,49
Butyric acid (butanoic acid)	Crumb, crust	Sweaty, rancid	3,7,8,10,16,19,30,33,36,41,42,43,45,49
3-Methylbutanoic acid (isovaleric acid)	Crumb, crust	Sweaty	3,7,8,10,11,12,30,32,33,36,41,42,43,45
Hexanoic acid (caproic acid)	Crumb, crust	Sweaty, cheesy, fatty, goat-like	7,8,11,12,14,16,26,41,44,45
2-Methylbutanoic acid	Crumb, crust	Cheese, rancid, sweaty	3,10,26,32,36,41,45
2-Methylpropanoic acid (isobutyric acid)	Crumb (traces), crust (traces)	Sweaty, butter, fatty, sour, rancid	7,8,11,16,26,30,32,33,42,43,45
Propionic acid (propanoic acid)	Crumb, crust	Rancid, pungent	3,7,8,11,16,31,33
Octanoic acid	Crumb, crust	Cheese, fatty, sweaty, soapy	12,14,16,23,26,41
Pentanoic acid	Crust	Sweaty	3,12,16,26,32
Lactic acid (2-hydroxypropanoic acid)	Crumb	Slight no unpleasant odour	14,16,33,34,44
Phenylacetic acid	Crumb, crust	Honey-like	3,41,43,45
Decanoic acid	Crumb, crust	Rancid, fatty, citrus, sweaty, cheesy	16,26,41
Heptanoic acid	Crumb, crust	Cheese, fatty, sweaty	23,26
Formic acid	[b]Bread	Pungent	16
Benzoic acid	[b]Bread	Faint	16
2-Oxopropanoic acid	[b]Bread	Faint	16
4-Oxopentanoic acid	[b]Bread	Caramellic	16
Pentanoic acid	Crust	Sweaty	41
Nonanoic acid	Crumb, crust	Cheese, fatty, sweaty	26
Lauric acid (dodecanoic acid)	[b]Bread	Bay oil	16, 23

[a]nf = not found in literature.

[b]Bread = crumb and crust were analysed together (mixed) in the mentioned article. Therefore, there is no knowledge if the volatile compound came from the crumb or crust.

[c] The numeration corresponds with the footnotes of Table 2A.

Table 7A. Volatile heterocyclic compounds found in wheat bread (crumb and/or crust) reported in literature their typical odours. Volatile compounds are in descending order by times cited.

Heterocyclic compounds	Crumb / Crust	Odour	References [c]
Furfural (2-furaldehyde)	Crumb, crust	Almond, bread-like, soil, burnt roasted, sweet, toasted	1,5,7,8,11,12,14,16,22,23,26,29,30,31,32, 33,42,44,47,49,50
2-Acetyl-1-pyrroline	Crumb, crust	Corn chip, roasty, crust-like, sweet, cereal, porpcorn-like, bread	9,10,13,16,21,29,36,37,41,43,45,47
Furfuryl alcohol	Crumb, crust	Burnt, warmy oil, mild	7,8,11,16,23,26,30,32,42,49
2-Acetylfuran	Crumb, crust	Smoky, roasty	7,8,16,22,26,32,41,47,49
2-Pentylfurane	Crumb, crust	Butter, green bean, floral, fruity, mushroom, raw nuts	1,2,5,6,7,11,26,29,49
3-Ethyl-2,5-dimethylpyrazine	Crust	Baked potato-like, earthy	13,16,22,26,41,45,47
3-Hydroxy-2-methyl-4-pyrone (maltol)	Crumb (traces), crust	Warm-fruity, caramellic-sweet	7,16,22,23,26,38,43
γ-Nonalactone	Crumb, crust	Coconut-like, sweet, fruity	5,13,26,30,41,45
4-Hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol)	Crumb, crust	Caramel, strawberry	9,10,26,36,41,45
2-Ethyl-3,5-dimethylpyrazine	Crumb, crust	Earthy	9,26,36,41,43
2,3,5-Trimethylpyrazine	Crust	Potato-like, earthy	1,5,22,41,47
Acetylpyrazine	Crust	Biscuit, cracker-like, crust-like, sweet, roasted	16,22,37,41,47
2-Methylpyrazine	Crumb, crust	Roasted, burnt, sweet	7,8,16,26,49
Pyrazine	Crust	Roasted	7,8,26,32
2-Methylfuran	Crust (traces)	Chocolate	7,11,16,26
2-Ethylpyrazine	Crumb, crust	Popcorn, nutty	7,16,26
2-Ethyl-3-methylpyrazine	Crumb, crust	Nutty, roasted, sweet	7,16,22,26
2,5-Dimethylpyrazine	Crust	Crust-like, popcorn	7,26,32
2-Acetylpyrrole	Crumb, crust	Roasted, biscuits	8,23,26

Table 7A.(continued)

Heterocyclic compounds	Crumb / Crust	Odour	References [c]
3-Ethyl-2- methylpyrazine	Crust	Roasted, burnt	37,41,47
3-Ethyl-2,6-dimethylpyrazine	Crust	Cooked potato	13,22,47
2-Acetyl-pyridine	Crumb, crust	Biscuit, cracker-like, crust-like, roasted	22,26,47
Pyrrole	Crust	Burnt, sweet	7,16,49
Butyrolactone	Crumb, crust	Creamy type	12,14,32
5-Ethyl-(3H)-furan-2-one	Crust	Spicy	22, 16,47
Dihydro-2-methyl-3 (2H)-furanone	Crumb	Spicy, rancid, butter	7,16,49
6-Acetyltetrahydropyridine	Crust	Roasty	36,45
2,6-Dimethylpyrazine	Crust	Roasted	8,26
2-Ethyl-6-methylpyrazine	Crust	Nutty	7,26
1-Methylpyrrol	Crust	Woody	16,26
2,3-Diethyl-5-methyl-pyrazine	Crumb, crust	Earthy	26,41
2-Ethyl-5-methylpyrazine	Crumb, crust	Baked	7,26
2-Acetyl-2-thiazoline	Crust	Biscuit, cracker-like, roasted, corn-chip	9,41
2,3-Dimethylpyrazine	Crumb, crust	Porcorn, roasted	26,32
2-Acetyl-3-hydroxyfuran	Crust	Caramel-like	22,26
5-Methyl-5H-cyclopenta-[b]-pyrazine	Crust	Roasted	37,47
2-Methyltetrahydro-3-furanone	Crumb, crust	Caramel-like	11,32
5-Ethyl-(5H)-furan-2-one	Crust	Spicy	16,47
3- Hydroxy- 4,5- dimethyl - 2(5H) furanone	Crumb	Spicy	3,36
3-Hydroxy-2-pyran-4-one	Crust	Sweet, caramel-like	41,43
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furanone	Crumb, crust	Caramel	9
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	Crumb, crust	Caramel	43
δ-Decalactone	[b]Bread	Caramel	43
5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone	Crust	Sweet, maple, caramel	36
2,5-Dimethyl-3(2H)-furanone	Crumb, crust	[a]nf	16
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	Crust	Caramelised	43
Furanone	Crust	Caramellic	45

Table 7A.(continued)

Heterocyclic compounds	Crumb / Crust	Odour	References [c]
δ-Dodecalactone	[b]Bread	Fruity	23
γ-Valerolactone	[b]Bread	Herbal	23
Ethyl maltol	Crumb, crust	Caramellic	23
5-methyl-2-(5H)-furanone	Crust	[a]nf	29
δ-Hexalactone	[b]Bread	Tonka type	23
2-Cyclohexanone	[b]Bread	Peardrop sweets	23
2-Cyclopenten-1-one	[b]Bread	[a]nf	16
Furan	[b]Bread	Rose	16
3-Methylfuran	Crust	Chocolate	29
2-Butylfuran	Crust	Green	26
2-Ethyl-benzofuran	Crust	[a]nf	29
2-Propenyl-2-furan	Crust	[a]nf	29
2-Phenylfuran	[b]Bread	[a]nf	16
Pyridine	[b]Bread	Rotting fish	32
2-Acetyl-tetrahidropyridine	Crumb, crust	Roasty, cracker-like	21
N-acetyl-4(H)-pyridine	Crust	Vegetable, roasted, biscuits	26
2,6-Diethylpyrazine	Crust	Green, spicy	26
2-Acteyl-1,4,5,6-tetrahydropyridine	Crust	Roasted	47
Vinylpyrazine	Crumb, crust	Boiled potato, earthy	26
2,3-Dimethylpyrazine	Crumb, crust	Popcorn, roasted	16
2,5-Dimethylpyrazine	Crust	Crust-like, popcorn	16
2-Propylpyrazine	Crumb, crust	Green, nutty, roasted, hot milk	26
2-Ethyl-3,6-dimethylpyrazine	Crumb, crust	Earthy	43
2-Methyl-3,5-diethylpyrazine	Crumb (traces), crust	Roasted, peanut butter	26
2-Propionyl-2-thiazole	Crust	Roasty	41
2-Acetyl-2-thiazole	Crust	Roasty	41
Diethylmethylpyrazine	Crust	Potato-like	47
2-Methoxy-3-isopropylpyrazine	Crumb	Earthy	21
2-Formyl-5-methyl-thiophene	Crust	Rancid, fatty, grass	26
2-Cyclohexanone	[b]Bread	Peardrop sweets	23

Table 7A.(continued)

Heterocyclic compounds	Crumb / Crust	Odour	References [c]
2-Isoamyl pyrazine	Crust	[a]nf	26
1-(2-Furanylmethyl)-pyrrole	Crust	Vegetable	26
2,3-Diethylpyrazine	Crust	Peanuts, roasted, earthy, coffee	26
2,5-Diethylpyrazine	Crumb, crust	[a]nf	26
2-Isobutyl-3-methylpyrazine	Crust	Caramellic	26
5-Methyl-2-vinylpyrazine	Crust	[a]nf	26
2-Methyl-6-(1-propenyl)-pyrazine	Crust	[a]nf	26
(1-Methyl-ethenyl)pyrazine	Crust	[a]nf	26
2-Isoamyl-6-methylpyrazine	Crust	Meaty	26
Indole (benzopyrrole)	Crumb, crust	Animal	26
Benzothiazole	Crumb, crust	Sulfurous	26
2-Methylpyrrole	[b]Bread	Woody	7
1-Furfuryl-pyrrol	[b]Bread	Vegetable	16
5-Hydroxymethyl-2-furaldehyde	[b]Bread	Butter, caramel, musty	16
2-Furandialdehyde	[b]Bread	[a]nf	16
2-Methyl-6-propyl-pyrazine	[b]Bread	Burnt, butterscotch	16
3-Methyl-3-ethyl-pyrazine	[b]Bread	[a]nf	16
2-Formylpyrrol	[b]Bread	Musty	16
2-Acetylpyrrol	[b]Bread	Musty	16
Butylated hydroxyanisole	[b]Bread	Phenolic type	23
3-Acetylthiophene	[b]Bread	Sulfurous	16

[a]nf = not found in literature.

[b]Bread = crumb and crust were analysed together (mixed) in the mentioned article. Therefore, there is no knowledge if the volatile compound came from the crumb or crust.

[c] The numeration corresponds with the footnotes of Table 2A.

Table 8A. Volatile alkanes, benzene and sulphur compounds found in wheat bread (crumb and/or crust) reported in literature their typical odours. Volatile compounds in each chemical group are in descending order by times cited

Volatile compound	Crumb / Crust	Odour	References [c]
Alkanes			
Limonene	Crust (traces)	Citrus	1,26,31,32
Octane	[b]Bread	Alkane	11,16
Hexane	Crumb, crust	Citrus	16
Decane	[b]Bread	Alkane	11
Tridecadien	[b]Bread	[a]nf	38
p-Cymene	Crumb, crust	Citrus	26
2-methylisoborneol	[b]Bread	Musty	39
Linalol	Crumb	Sweet-floral	26
β-myrcene	Crumb	Spicy	31
α-Pinene	Crumb	Herbal	31
Sabinene	Crumb	Woody	31
1,1-diethoxyisopentane	[b]Bread	[a]nf	38
Benzene compounds			
Ethylbenzene	Crumb	Gasoline	30
Styrene	Crumb	Pungent	30
Toluene	Crumb	Sweet, pungent	32
1,2-Dimethylbenzene	Crumb	Sweet	30
1,4-Dimethylbenzene	Crumb	Sweet	30
1,2-Ethylmethylbenzene	Crumb	[a]nf	30
1,4-Ethylmethylbenzene	Crumb	[a]nf	30
Trimethylbenzene	Crumb	Oily	30
1,2-Dichlorobenzene	Crumb	[a]nf	30
Methylbenzene	Crumb	Sweet, pungent,	1
Butyrated hydroxytoluene	[b]Bread	[a]nf	23
Nitrobenzene	[b]Bread	Almond-like	38
Sulphur compounds			
Dimethyltrisulphide	Crumb, crust	Cabbage-like	31,41,45
Methyl trisulfide	Crumb	[a]nf	32
Dimethyl disulphide	Crumb, crust	Garlic	16, 31
Methanethiol	Crust	Rotting cabbage	16,41,45
Dimethyl sulphide	[b]Bread	Cabbage-like	16
3-Butenyl-isothiocyanate	[b]Bread	Aromatic pungent	32

[a]nf = not found in literature.

[b]Bread = crumb and crust were analysed together (mixed) in the mentioned article. Therefore, there is no knowledge if the volatile compound came from the crumb or crust.

[c] The numeration corresponds with the footnotes of Table 2A.