Analytical Methods

Determination of α-keto acids in pork meat and Iberian ham via tandem mass spectrometry

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An analytical method which offers accurate determination and identification of eight α-keto acids (α-ketoglutaric acid, pyruvic acid, 4-hydroxyphenylpyruvic acid, 3-methyl-2-oxobutyric acid, α-keto-γ-methylthiobutyric acid, 4-methyl-2-oxovaleric acid, 3-methyl-2-oxovaleric acid, and phenylpyruvic acid) in pork meat and Iberian ham samples is reported. The method utilises a highly selective and sensitive method of multiple reaction monitoring (MRM) by mass spectrometry. The analytical method is simple (although the chemical derivatisation of the α-keto acids with dansylhydrazine is required), precise (<18% RSD), accurate (90–110%), sensitive (0.01–0.34 mg/kg of defatted and freeze–dried meat depending on the α-keto acid) and linear (R > 0.99) over several orders of magnitude (until 0.01–146.1 mg/kg of defatted and freeze–dried meat depending on the α-keto acid). Using this methodology, α-keto acids were found to be present in pork meat to a low extent, and their concentration increased when they were determined in Iberian ham. This is the first report of the presence of α-keto acids in both pork meats and Iberian hams.

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1. Introduction

The aromas of food products are determined by unique combinations of volatile compounds. The most important aroma compounds include esters, terpenes, benzene derivatives, and amino acid and lipid-derived compounds, among others, which will be produced to different extents depending on the food composition and the chemical or biochemical reaction pathways involved.

Amino acid catabolism has been shown to play a major role in the aroma of fermented food products. In these products, as well as in other food products, amino acid catabolism is initiated by a transamination reaction that produces α-keto acids (Gonda et al., 2010; Ziadi et al., 2010). The α-keto acids resulting from transamination can be further transformed into aldehydes by a keto acid decarboxylase or into carboxylic acids by a keto acid dehydrogenase.

Nevertheless, different studies have pointed out to the alternative possibility that α-keto acids can also be produced and decomposed chemically. Thus, Zamora, Navarro, Gallardo, and Hidalgo (2006) found that α-amino acids could be converted into α-keto acids as a consequence of their reaction with the lipid oxidation product 4,5-epoxy-2-decenal. Moreover, Smit et al. (2004) showed that α-keto acids can be degraded chemically, and this degradation play a role in flavor formation in fermented foods. Additionally, α-keto acids can also react with other compounds present in the food to produce further reactions (Zamora, Delgado, & Hidalgo, 2011).

Nevertheless, due to the complexity of food matrixes and lack of this kind of studies is likely a consequence of the absence of completely satisfactory and sensitive procedures that can determine α-keto acids at low concentration in complex food matrixes, which is due to the high instability and polarity of the α-keto acids being examined (Fuschs et al., 2009). Nowadays, α-keto acids are mostly determined after reaction with α-phenylenediamine and 2-mercaptoethanol to form fluorescent quinoxalinol derivatives, which are separated by HPLC and quantified after fluorimetric detection (Ferreira, Reis, Rodrigues, Oliveira, & de Pinho, 2007; Fuschs et al., 2009). However, this method has some drawbacks. Thus, the modification of α-keto acids in the presence of α-phenylenediamine is carried out in acidic aqueous conditions. Under these conditions, amino acids are non-enzymatically degraded to α-keto acids. Therefore, the common presence of some amino acids at this stage has an adverse effect on the accuracy of the assay (Kato, Kito, Hemmi, & Yoshimura, 2011). As an alternative, an organic solvent has been recently proposed to be used instead of an aqueous solution to avoid this problem (Kato et al., 2011). Nevertheless, due to the complexity of food matrixes and...
the low extent to which α-keto acids are expected to be present in non-fermented foods, the use of DAD detection, or even fluorimetric detection, may not always be adequate for the determination of these compounds.

Mass spectrometric detection has the advantage of providing precise structural information about the eluted compounds since co-elution is not a problem as long as they have different molecular masses. In addition, sensitivity and selectivity of detection can be further increased using tandem MS. Although HPLC–MS/MS offers a number of key advantages for the analysis of food components (see, for example, Hull, Woodside, Ames, & Cuskelley, 2012; Onal, 2011; Pena-Gallego, Hernandez-Orte, Cacho, & Ferreira, 2012), to our best knowledge there is not report yet on the use of this coupling for determining α-keto acids in foods, although the identity of the quinoxalinol derivatives of four α-keto acids in wine was confirmed by LC–MS/MS (Ferreira et al., 2007).

The aim of this study was to develop the first LC–MS/MS analytical method for the determination of α-keto acids in foods. The method is based on the derivatisation of α-keto acids with dansylhydrazine using trifluoromethanesulphonic acid as catalyst. The developed method is simple, robust, and accurate. It has been applied to the determination of α-keto acids in pork meat and Iberian ham.

2. Experimental

2.1. Materials

The commercial α-keto acid standards used in this study were: α-ketoglutaric acid (GlukA, α-Keto Acid derived from Glutamic acid), pyruvic acid (AlaK, α-Keto Acid derived from Alanine), 4-hydroxyphenylpyruvic acid (TyrK, α-Keto Acid derived from Tyrosine), 3-methyl-2-oxobutyric acid (ValA, α-Keto Acid derived from Valine), α-keto-γ-methylthiobutyric acid (MetA, α-Keto Acid derived from Methionine), 4-methyl-2-oxovaleric acid (LeuK, α-Keto Acid derived from Leucine), 3-methyl-2-oxovaleric acid (IleK, α-Keto Acid derived from Isoleucine), phenylpyruvic acid (PheK, α-Keto Acid derived from Phenylalanine), and 2-oxovaleric acid (IS, the internal standard employed in the developed procedure). They were obtained from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Acetonitrile and methanol were of HPLC gradient grade obtained from Merck (Darmstadt, Germany). Stock solutions of α-keto acids were prepared with methanol. Diluted mix standards were also prepared with methanol. All solutions were filtered through 0.45 μm membranes (Millipore) and degassed prior to use.

2.2. Instrumentation and optimisation

Samples were analysed using Agilent liquid chromatography system (1200 Series) consisting of binary pump (G1312A), degasser (G1379B), and autosampler (G1329A), connected to a triple-quadrupole API 2000 mass spectrometer (Applied Biosystems) using an electrospray ionisation interface in positive ionisation mode (ESI+). Compounds were separated on a Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5 μm) column from Agilent. As eluent A, a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate was used. As eluent B, a 0.2% formic acid solution in acetonitrile was employed. The mobile phase was delivered at 0.5 mL/min in linear gradient mode: 0 min 0% B, 40 min 30% B, 45 min 100% B, 50 min 100% B, 51 min 0% B, 60 min 0% B. This gradient was selected because it provided the effective separation of the eight α-keto acids and the IS. Mass spectrometric acquisition was performed by using multiple reactions monitoring (MRM). The nebuliser gas (synthetic air), the curtain gas (nitrogen), and the heater gas (synthetic air) were set at 45, 30, and 50 (arbitrary units), respectively. The collision gas (nitrogen) was set at 3 (arbitrary units). The heater gas temperature was set at 500 °C and the electrospray capillary voltage to 5.5 kV. The focusing potential was 370 V, and the declustering potential was 21 V. The fragment ions in MRM mode were produced by collision-activated dissociation of selected precursor ions in the collision cell of the triple quadrupole and analysed the selected products with the second analyser of the instrument. Three transitions were acquired for the identification of each dansylhydrazone derivative.

To establish the appropriate MRM conditions for the individual compounds, the mass spectrometric conditions were optimised using infusion with a syringe pump to select the most suitable ion transitions for the target analytes. Precursor and product ions used for quantification and confirmation purposes, and operating conditions are summarised in Table 1.

2.3. Standard curve and internal standard preparation

A 10 mM α-keto acids stock solution in methanol was serially diluted with methanol to prepare a 10-point standard curve and added to 100 mg of a defatted and freeze–dried pork meat sample having a very low α-keto acid content. α-Keto acids were extracted, derivatised, and determined as described in Section 2.5. Additionally, the same concentrations of the α-keto acids were also derivatised and determined in the absence of the meat matrix as described in Section 2.4. A 0.25 nmol/mL 2-oxovaleric acid (IS, internal standard) working solution was prepared in methanol.

2.4. Derivatisation of α-keto acids

α-Keto acids were derivatised previously to their separation to form products with improved mass spectrometric properties. The corresponding dansylhydrazones were formed by mixing a solution of α-keto acids in methanol (100 μL) with 50 μL of IS, 30 μL of trifluoromethanesulphonic acid solution (3% in methanol), and 200 μL of dansylhydrazine solution (2 mg/mL in methanol) (Appelblad, Pontén, Jaegfeldt, Bäckström, & Irgum, 1997; Hyytiainen et al., 1996). The resulting solution was kept at 25 °C for 1 h, then, diluted with 1 mL of eluent A (a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate), and analysed by LC–MS/MS.

2.5. Sample preparation

Meat samples (50 g) were triturated and extracted twice with 100 mL of hexane with agitation at room temperature (22 °C) for 30 min to remove non-polar lipids. The hexane was removed and the defatted meat was frozen and freeze–dried. Lyophilised meat (100 mg) was treated with 50 μL of internal standard solution (0.25 nmol/mL of α-oxovaleric acid in methanol) and extracted with 5 mL of methanol. The suspension was stirred for 3 min at room temperature and centrifuged (5 min at 2000g). The supernatant was collected and taken to dryness under nitrogen. The obtained residue was dissolved in 150 μL of methanol, and treated successively with 30 μL of trifluoromethanesulphonic acid solution (3% in methanol) and 200 μL of dansylhydrazine solution (2 mg/mL in methanol). The resulting solution was kept at 25 °C for 1 h, then, diluted with 1 mL of eluent A (a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate), and analysed by LC–MS/MS.

2.6. Statistics and method validation

Calibration curves were constructed from the peak area ratios of the different analytes to the IS versus the theoretical
concentrations. Linear regression analysis of the standard curves and calculation of α-keto acid concentrations was undertaken using Origin® 7.0 (OriginLab Corporation, Northampton, MA). The method precision was presented as the relative standard deviation (RSD) of replicate analyses. The accuracy of the method was expressed as (mean calculated concentration)/(spiked concentration) × 100. The limit of detection (LOD) was defined as the lowest sample concentration that could be detected with a signal-to-noise ratio (S/N) greater than five (Colgrave, Allingham, & Jones, 2008). This criterion was employed because of the complexity of the matrix, although a signal-to-noise ratio greater than three is accepted to alkyl catalysis rather than acid catalysis (Appelblad et al., 1997; Hyytiaînen et al., 1996) and aldehydes (Al-Dirbashi et al., 2006; Baños & Silva, 2009), among others. Because α-keto acids have a carbonyl group, their derivatisation with dansylhydrazine is easily carried out. To obtain satisfactory results, the reaction is better carried out in the absence of water because this reaction is subjected to alkyl catalysis rather than acid catalysis (Appelblad et al., 1997). This is not an inconvenient when working with α-keto acids because α-keto acids are extracted from the meat matrix with methanol (Mühling, Paddenberg, Hempelmann, & Kummer, 2006) and the dansylation reaction is carried out in the same solvent.

Standard solutions of α-keto acid dansylhydrazones were employed for the determination of the optimum conditions of MRM. MS in positive ESI were dominated by [M+H]+ ions and these ions were selected for collision-activated dissociation studies of the precursor ions. For each analysed α-keto acid, three transitions were monitored. The first one was employed for quantitation (the first MRM transition in Table 1) and the other two were employed to confirm the identity of the α-keto acid. It has been demonstrated that optimisation of the ESI parameters play a role in the achievement of adequate MS signals for any transition (Biesaga & Pyrzynska, 2009; Mullen, Boitier, Steward, & Crozier, 2004). Therefore, declustering potential (DP), focusing potential (FP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and collision cell exit potential (CXP), were optimised in infusion mode for each transition. The voltage required for significant fragmentation was compound dependent parameter (Table 1).

For most α-keto acid dansylhydrazones, the MRM method was built on the formation of different ions derived from the dansylhydrazone group. The main product ion for most analysed dansylhydrazones was 5-hydrosulfonyl-N,N-dimethylnaphthalen-1-aminium (m/z 236.1).

### Table 1

Optimisation of MRM transitions for detection of α-keto acids.

<table>
<thead>
<tr>
<th>α-Keto acid Abbreviation</th>
<th>Monitored transition</th>
<th>DP</th>
<th>FP</th>
<th>EP</th>
<th>CEP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ketoglutaric acid GluKA</td>
<td>394 → 170.0</td>
<td>21</td>
<td>370</td>
<td>10.0</td>
<td>16</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>3-Methyl-2-oxovaleric acid IleKA</td>
<td>378 → 170.1</td>
<td>21</td>
<td>370</td>
<td>9.5</td>
<td>16</td>
<td>89</td>
<td>4</td>
</tr>
<tr>
<td>4-Methyl-2-oxovaleric acid LeuKA</td>
<td>378 → 170.1</td>
<td>21</td>
<td>370</td>
<td>9.5</td>
<td>16</td>
<td>53</td>
<td>6</td>
</tr>
<tr>
<td>2-Oxovaleric acid IS</td>
<td>364 → 236.1</td>
<td>26</td>
<td>370</td>
<td>10.0</td>
<td>16</td>
<td>31</td>
<td>6</td>
</tr>
</tbody>
</table>
3.2. Optimisation of the derivatisation reaction

The acid catalyst employed for the derivatisation reaction and its concentration is known to be the factor most strongly affecting the formation of dansylhydrazones (Appelblad et al., 1997). Therefore, different concentrations of trifluoromethanesulphonic acid were assayed in order to determine the reaction time and the stability of the produced compound. Fig. 1 shows the behaviour of some selected derivatives formed by employing two concentrations of the acid: 3% and 10%. As observed in the figure, the different \( \alpha \)-keto acids exhibited different behaviours. For example, the derivative of AlaKA was rapidly formed and decomposed when 10% trifluoromethanesulphonic acid solution in methanol was employed. On the contrary, although it was also rapidly formed, the derivative resulted much more stable when 3% solution of the acid was employed. Differently to AlaKA, the formation of the derivative of ValKA was produced more slowly and a higher response was obtained with 10% trifluoromethanesulphonic acid solution in methanol than when 3% solution was employed. However, when a 10% solution of the acid was employed, a stable response for the derivative as a function of time was not obtained. On the contrary, although a lower response was obtained when 3% acid solution was employed, this response was stable for at least 6 h. Analogous results were also observed for the different compounds analysed, but in some cases an incubation time of 1 h was needed to form the derivative. For that reason, derivatives were prepared using 3% trifluoromethanesulphonic acid solution in methanol and the reaction maintained for 1 h at 25 \( ^\circ \)C to assure complete formation of the dansylhydrazones of all \( \alpha \)-keto acids. These derivatives should be analysed within the next 6 h after preparation to ensure quantitative results. Using these conditions, the precision of the determination of the different \( \alpha \)-keto acids using standards was satisfactory. The RSD were in the range of 2.8–7.4% for the studied compounds.

3.3. Development of the LC–MS method

A LC method was developed to elute sequentially from the column the different derivatives. Special attention was paid to two pairs of compounds: ValKA/IS and IleKA/LeuKA, because of their identical molecular weights and selected MRM transitions (see Table 1). Using a Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5 \( \mu \)m) column and gradient elution, the dansylhydrazones of the eight \( \alpha \)-keto acids and the IS were separated in less than 40 min. The elution sequence was directed by their molecular weights and structural characteristics. In general, more polar and smaller compounds eluted earlier than larger and less polar compounds (Fig. 2). For this reason, GluKA was the first eluted compound, TyrKA appeared much earlier than PheKA, and the pair ValKA/IS appeared earlier than the pair IleKA/LeuKA. In addition, using the developed procedure, ValKA could be separated from the IS, and IleKA could be separated from LeuKA.

Depending on the reaction conditions, the reaction of \( \alpha \)-keto acids with dansylhydrazine usually yields two geometric isomers which are separated by HPLC. In the present study, the presence of both isomers was observed in all assayed compounds with the exception of ValKA (Fig. 2). Nevertheless, the area of one isomer was always much higher than the area of the other. Because the ratio of the two peaks does not depend on the concentration of the carboxylic compound involved (Nondek, Rodier, & Birks, 1992), only the area of the larger peak was used for quantification for the different \( \alpha \)-keto acids with the exception of IleKA. Under the employed conditions, the dansylation reaction of this last \( \alpha \)-keto acid produced two isomers that appeared very close in the
chromatogram and could not be satisfactorily separated. The total area of both peaks was used for the quantification of IleKA.

3.4. Calibration curve

Six sets of standards were prepared by spiking a defatted and freeze–dried pork meat sample, having a very low α-keto acid content, with the eight α-keto acids and adding a known amount of IS (2-oxovaleric acid). The pork meat selected for spiking the standards was the pork meat that had the lowest α-keto acid content among the different pork meats assayed. 2-Oxovaleric acid was selected as the IS because it is available commercially, has similar chemical properties to the analytes, and, after derivatisation, possesses similar ionisation and chromatographic properties. Each sample was analysed on triplicate (on three different days) to determine the dynamic range, limit of detection, precision and accuracy for the method. Assays were linear (R > 0.99) in the concentration range 0.1–200 mg/kg of defatted and freeze–dried meat (1–1000 mg/kg of defatted and freeze–dried meat for GluKA and AlaKA). LODs were ranged between 0.1 and 2.1 mg/kg of defatted and freeze–dried meat for GluKA and PheKA, respectively (Table 2). Calibration standards prepared in methanol showed relative standard deviations (RSD) of <8% (see above), while calibration standards in which α-keto acids were spiked into a defatted and freeze–dried pork meat had an RSD of less than 18% and were accurate within 10% of the theoretical concentrations (Table 2). The increased variability in these samples was owing to the endogenous level of α-keto acids in these samples. Ion suppression effects in the meat and ham samples were observed and required the use of spiked standards rather than pure standards.

The intra- and inter-assay accuracy and precision data for the quality control meat samples are presented in Table 2. The precision is given by the relative standard deviation (RSD), while the accuracy was calculated by comparing the concentration of the meat sample spiked with a known amount of α-keto acids to the theoretical value after background subtraction of the endogenous α-keto acid levels. For all quality control samples, RSD values <15% (usually <10%) were achieved in the intraday assay and calculated concentrations were usually within 10% of the theoretical value. RSD values increased slightly in the interassay, in which they were <18%. In general, the precision of the method was better for MetKA and LeuKA (RSD < 8%) than for GluKA, TyrKA, ValKA, IleKA and PheKA (RSD < 15%). The worst results were obtained for AlaKA (RSD 18.1%),

3.5. Characterisation of meat and Iberian ham α-keto acids

To our best knowledge, there is not previous report indicating the presence of α-keto acids in either pork meat or Iberian ham. Nevertheless, the presence of these compounds can be easily determined by following the procedure described in the present study. This study determined the α-keto acid content of five pork meats and five Iberian hams from different origins. The chromatograms of the extracts of one representative pork meat and one representative Iberian ham are presented in Fig. 3. Peak identity was established by both the retention time compared to that of authentic standards and the characteristic transitions (precursor and product ion pairs). The results of the quantitative determination of α-keto...
Table 3

Content of α-keto acids in pork meat and Iberian ham samples.

<table>
<thead>
<tr>
<th>α-keto acid</th>
<th>Pork meat</th>
<th>Iberian ham</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluKA</td>
<td>2.07 (1.02–3.33)</td>
<td>19.91 (13.37–35.50)</td>
</tr>
<tr>
<td>AlaKA</td>
<td>26.96 (11.54–34.45)</td>
<td>46.06 (36.96–68.28)</td>
</tr>
<tr>
<td>TyrKA</td>
<td>0.26 (&lt;0.16–0.42)</td>
<td>0.86 (0.50–1.40)</td>
</tr>
<tr>
<td>MetKA</td>
<td>0.18 (&lt;0.16–0.33)</td>
<td>1.42 (0.95–1.70)</td>
</tr>
<tr>
<td>IleKA</td>
<td>1.24 (0.38–2.13)</td>
<td>4.71 (2.73–10.87)</td>
</tr>
<tr>
<td>LeuKA</td>
<td>1.63 (0.48–2.55)</td>
<td>9.28 (6.22–14.12)</td>
</tr>
<tr>
<td>PheKA</td>
<td>0.95 (&lt;0.34–2.41)</td>
<td>9.77 (5.57–13.82)</td>
</tr>
</tbody>
</table>

All values: mean (range) are given as mg/kg of defatted and freeze–dried meat (or ham). Five samples of meat and Iberian ham from different origins were analysed (n = 3 for each sample, RSD < 18%).

4. Conclusions

The LC–MS/MS method detailed in this report allows a sensitive and reproducible separation and quantification of eight α-keto acids, which have been described for the first time in pork meat and Iberian ham. Since the values for the limit of detection and quantification are low in the relation to the usual content of α-keto acids in meat and Iberian ham samples, it could be reasonable to conclude that this method can be used for reliable quantitative analysis of α-keto acids in muscle samples without preconcentration step.

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