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Analytical Methods

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

Francisco J. Hidalgo, José L. Navarro, Rosa M. Delgado, Rosario Zamora *

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Avenida Padre García Tejero 4, 41012 Seville, Spain

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ABSTRACT

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).

The possibility that lipid oxidation can produce the conversion of α-amino acids into α-keto acids has not been yet satisfactorily addressed in food products. However, in many of these products, lipid oxidation is routinely produced as a part of the elaboration process. Therefore, α-keto acids might be being formed as a consequence of these processes.

The 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 *o*-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 *o*-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

* Corresponding author. Tel.: +34 954 611 550; fax: +34 954 616 790.
E-mail address: rzamora@ig.csic.es (R. Zamora).

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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, & Cuskelly, 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 (AlaKA, α-Keto Acid derived from Alanine), 4-hydroxyphenylpyruvic acid (TyrKA, α-Keto Acid derived from Tyrosine), 3-methyl-2-oxobutyric acid (ValKA, α-Keto Acid derived from Valine), α-keto-γ-methylthiobutyric acid (MetKA, α-Keto Acid derived from Methionine), 4-methyl-2-oxovaleric acid (LeuKA, α-Keto Acid derived from Leucine), 3-methyl-2-oxovaleric acid (IleKA, α-Keto Acid derived from Isoleucine), phenylpyruvic acid (PheKA, 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 (S. 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, derivatisated, and determined as described in Section 2.5. Additionally, the same concentrations of the α-keto acids were also derivatisated 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 derivatisated 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; Hyytiaïnen 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 2000*g*). 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

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