Development of a UPLC-MS/MS method for establishing Serra da Estrela cheese's free amino acids profile

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INTRODUCTION

- ✓ ***Serra da Estrela*** is a high-value and widely appreciated Portuguese cheese, which as a Protected Designation of Origin (PDO) recognition, being its production legally regulated.
- ✓ The amino acids composition (namely the essential amino acids), plays a fundamental role on the nutritional and technological value of cheese, influencing greatly its flavor.
- ✓ In this work, a mass chromatographic method was developed and applied for assessing the amino acids profile of PDO ewe cheeses.
- ✓ Evaluate the variability of amino acids contents in PDO cheeses due to cheese producer and production date.

AIMS

To develop a UPLC–MS/MS method

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Photo-diode-array detector (DAD)

Mass detector equipped with an ESI source

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To establish

→ **Calibration curves for essential and non-essential amino acids**

→ **Limits of detection and quantification (LOD** and **LOQ)**

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To determine

→ **Amino acids levels of PDO Serra da Estrela cheeses**

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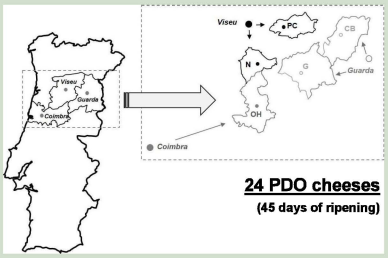
To evaluate

The existence of profile differences due to producer or production date

SAMPLES

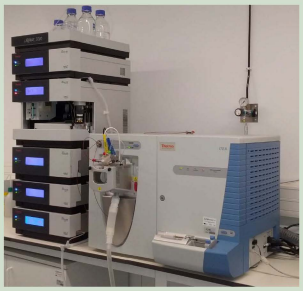


24 PDO cheeses
(45 days of ripening)

- ❖ **6 certified producers**: *Producers 1-6*
- ❖ **5 municipalities**: **CB** → *Celorico da Beira*
  G → *Gouveia;* **N** → *Nelas;* **OH** → *Oliveira do Hospital*
  PC → *Penalva do Castelo*
- ❖ **5 production dates:**
  2017: *November* and *December*
  2018: *January*, *February* and *March*

UPLC–MS/MS method

- ✓ Dionex Ultimate 3000 UPLC instrument with a quaternary pump, an autosampler (5 °C) and a degasser system
- ✓ Photo diode-array detector (DAD)
- ✓ MS detection (positive mode) by multiple reaction monitoring (MRM) using a Linear Ion Trap LTQ XL mass spectrometer equipped with an ESI source



- ✓ Mass spectra acquired from 100–1500 *m/z* with a collision energy of 14-30 (a.u.)
- ✓ Chromatographic separation:
  - → U-VDSpher PUR C18-E column (100mm×2.0 mm id, 1.8 µm), 40 °C
  - → Mobile phase, eluent A (0.1% (v/v) formic acid in water) and eluent B (0.1% (v/v) formic acid in acetonitrile/water (50:50, v/v)), multistep gradient at 0.40 mL/min and an injection volume of 5 µL
  - → He (50 psi), operation voltage of 5.5 kV, a source temperature of 400°C, a capillary voltage of 18 V and tube lens offset kept at 25 V
- ✓ Data acquisition: Xcalibur®data system

Amino acids detection/assessment:

Calibration standards:

0–7000 µmol/L of 22 amino acids in water (Milipore Milli-Q system, which includes a reverse osmosis, ion exchange and filtration steps), being ***N-Acetyl-L-Tyrosine used as internal standard***

Cheese samples:

5.0 g + 10 mL of water:acetonitrile (50:50, v/v) + 3.0 mM ***N-Acetyl-L-Tyrosine (internal standard)***

Mixture shaken in a vortex during 5 min, then sonicated for 10 min at room temperature (20 ºC) and immediately centrifuged at 10.000 RPM (4 ºC) during 10 min

The supernatant was filtered through 0.2 µm nylon membrane filter and stored at –4 ºC until analysis.

RESULTS

Free amino acids detection by UPLC–MS/MS (standards and cheese samples)

Table: Chromatographic and MRM parameters for free amino acids detection by UPLC–MS/MS and mean levels in ***Serra da Estrela*** cheeses

Amino acid	Retention time (min)	Quantification transition (*m/z*)	Confirmatory Transition (*m/z*)	Collision energy (V)	LOD (µmol/L)	LOD (mg/100g cheese, wb)	LOQ ((µmol/L)	LOQ (mg/100g cheese, wb)	Cheese samples $\bar{x} \pm s$ (mg/100g, wb)
Histidine	0.82	156	137, 111, 109, 94	25	0.36	*0.011*	1.10	*0.034*	0.18±0.09
Lysine	0.81	147	130, 129, 100	25	0.11	*0.003*	0.35	*0.010*	0.34±0.30
Glutamine	0.81	147	129, 100, 83	26	0.41	*0.012*	1.25	*0.036*	0.35±0.41
Glutamic acid	0.81	148	130, 129, 101, 83	25	0.18	*0.005*	0.54	*0.016*	2.0±2.7
Serine	0.58	106	88, 87, 85, 59	25	0.47	*0.010*	1.42	*0.030*	0.52±0.28
Alanine	0.85	90	68, 61	18	26.9	*0.479*	81.5	*1.452*	N.D.
Glycine	0.85	76	75, 47, 29	14	4.11	*0.062*	12.4	*0.187*	N.D.
Threonine	0.59	120	101, 99, 83, 73, 71, 55	25	0.32	*0.008*	0.96	*0.023*	0.27±0.19
Aspartic acid	0.60	134	115, 87,86, 73	15	0.14	*0.004*	0.42	*0.011*	0.79±0.57
Valine	0.67	117	100, 90, 71	25	0.15	*0.004*	0.47	*0.011*	9.1±5.1
Methionine	0.76	150	132, 103, 101, 55	25	0.18	*0.006*	0.56	*0.017*	1.1±0.8
Proline	0.81	132	85	20	0.50	*0.006*	1.53	*0.018*	33±12
Isoleucine + Leucine	0.82	132	120, 114, 104, 86, 85, 71, 68	25	0.16	*0.004*	0.50	*0.013*	40±15
Asparagine	0.82	133	115, 112, 104, 87, 89, 85	25	0.22	*0.006*	0.67	*0.018*	24±14
Arginine	0.62	175	157, 140, 130, 115, 111, 97	30	0.25	*0.009*	0.75	*0.026*	0.11±0.07
Phenylalanine	1.01	166	148, 130, 119	25	11.8	*0.391*	35.9	*1.185*	23±10
Tryptophan	1.46	205	187, 159, 132	25	0.12	*0.005*	0.36	*0.015*	2.2±1.7
Cysteine	0.60	121	98, 97, 75	25	1.28	*0.031*	3.88	*0.094*	75±27
Tyrosine	0.74	182	164, 135	25	0.47	*0.017*	1.42	*0.051*	1.0±1.4
Cystine	0.64	241	224, 14, 177, 168, 93, 151	22	0.33	*0.031*	0.99	*0.095*	N.D.

Green: essential amino acids; Blue: non-essential amino acids; N.D.: not detected
LOD: limit of detection (LOD=3.3×[intercept error/slope]; **LOQ**: limit of quantification (LOQ=10×[intercept error/slope]; **24 cheeses × 2 independent samples x 3 injections**
Cheese samples amino acids contents: mean ± standard deviation (mg/100g of cheese in *wet basis*) regarding 24 × 2 independent cheese samples, from 6 certified PDO Serra da Estrela producers, located in 5 municipalities within the PDO geographical region and produced at 5 different time-periods (5 production dates)

➢ ***Essential amino acids***

i. 9 amino acids detected (Leucine and Iso-leucine quantified together)

ii. Mean levels (*wet basis*): **75 ± 30 mg/100g** of cheese

iii. Minimum-maximum (*wet basis*): **19 to 167 mg/100g** of cheese

➢ ***Non-essential amino acids***

i. 9 amino acids detected (Alanine and Glycine not detected)

ii. Mean levels (*wet basis*): **136 ± 46 mg/100g** of cheese

iii. Minimum-maximum (*wet basis*): **44 to 262 mg/100g** of cheese

The observed variability pointed out that the ***Serra da Estrela*** cheese **amino acids levels** may be **influenced** by the **cheese producer** and by the **production date**

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Amino acids may be used, in the future, as ***possible origin biomarker***

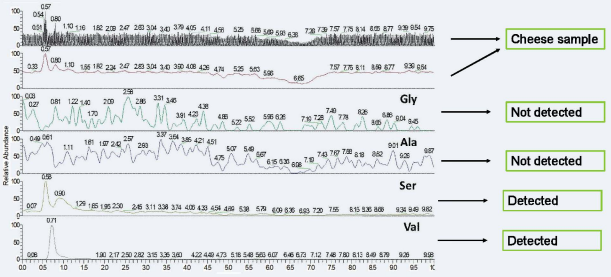

Figure. Example of a amino acids chromatographic profile of a cheese sample

CONCLUSIONS

- UPLC-MS/MS method was successfully applied for establishing the free amino acids profile and contents of Serra da Estrela PDP cheeses
- 18 amino acids could be quantified in all cheese samples, obtained from 6 cheese certified producers, located in 5 municipalities within the PDO geographical region, and produced during 5 months (November 2017 to March 2018)
- 100 g of chees (wb) may allow an intake of **75 ± 30 mg of essential amino acids**
- 100 g of chees (wb) may allow an intake of **136 ± 46 mg of non-essential amino acids**
- **The variability of the concentration levels found may foresee the future use of the amino acids profiles as a possible biomarker for cheese origin and/or production date**

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