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Short communication

Simultaneous determination of carotenoids, tocopherols, retinol and cholesterol in ovine lyophilised samples of milk, meat, and liver and in unprocessed/raw samples of fat

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ABSTRACT

An accurate, fast, economic and simple method to determine carotenoids, tocopherols, retinol and cholesterol in lyophilised samples of ovine milk, muscle and liver and raw samples of fat, which are difficult to lyophilise, is sought. Those analytes have been studied in animal tissues to trace forage feeding and unhealthy contents. The sample treatment consisted of mild overnight saponification, liquid-liquid extraction, evaporation with vacuum evaporator and redissolution. The quantification of the different analytes was performed by the use of ultra-high performance liquid chromatography with diode-array detector for carotenoids, retinol and cholesterol and fluorescence detector for tocopherols. The retention times of the analytes were short and the resolution between analytes was very high. The limits of detection and quantification were very low. This method is suitable for all the matrices and analytes and could be adapted to other animal species with minor changes.

1. Introduction

In addition to their essential roles and their properties beneficial to human health, carotenoids, retinol and tocopherols in animal tissues contribute to enlarge the shelf-life of the products and protect them against oxidation (Ripoll, González-Calvo, Molino, Calvo, & Joy, 2013). They have also been used to trace forage-feeding in ruminants as forages have greater contents than concentrates (Prache, Priolo, & Grolier, 2003). Cholesterol also plays an essential role in animals; however, elevated human plasma cholesterol concentration may increase the risk of cardiovascular diseases and atherosclerosis (Connor & Connor, 2002) and a maximum intake of cholesterol of 300 mg per day for adults is recommended (World Health Organisation, 2013).

In long-time experimental trials, which can last for months, serially samples can be obtained throughout the experimental period and are usually frozen to be analysed all together. Samples for carotenoids, tocopherols and retinol analyses should be stored at −80 °C because they are sensible to oxygen, direct light and temperature (Chen, Chen, & Chien, 1994). Storing unprocessed samples at −80 °C for months is inconvenient because of their bulkiness, which could be reduced storing lyophilised samples. However, lyophilisation of meat and milk is easy and it is a short process but that of fat seems unaffordable.

Carotenoids and tocopherols have been determined in ovine milk (Gentili et al., 2012), adipose tissue and liver (Alvarez, Melendez-Martinez, Vicario, & Alcalde, 2014; Yang, Larsen, & Tume, 1992) and muscle (Osorio, Zumalacarregui, Cabeza, Figueira, & Mateo, 2008). There are methods for fast determination of carotenoids and cholesterol in beef meat (Prates, Quaresma, Bessa, Fontes, & Alfaia, 2006) or carotenoids, retinol and tocopherol in bovine milk and serum (Chauveau-Duriot, Doreau, Noziere, & Graulet, 2010). But, to our knowledge they have not been adapted for ovine and for other tissues such as liver and fat. Therefore, the aim of this study was to implement a fast, accurate and simple method to determine carotenoids, tocopherols, retinol and cholesterol in ovine lyophilised meat, liver and milk and raw samples of fat.

2. Materials and methods

2.1. Reagents and standards

L-Ascorbic acid (99% purity) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) (≥99% purity) were purchased from Sigma-Aldrich (Sant Louis, Missouri, USA). Potassium hydroxide (90% purity), ethanol (96% purity), hexane (99% purity) and ethyl acetate (99.8% purity) were

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Table 1
Solvents, wavelength and molar extinction coefficients (ε) of tocopherols, retinol and carotenoids to determine the exact concentration and quality analysis (QA) of the standards.

	Solvent	λ (nm)	$\varepsilon_{1\,cm}^{1\%}$	Quality analyses[1]	Concentration (μg ml^{-1})
α-Tocopherol	Methanol	292	76	$A_{255nm}/A_{292\ nm} \leq 0.180$	$\frac{A_{292}\cdot 10^4}{76}$
γ-Tocopherol	Methanol	298	91	–	$\frac{A_{298}\cdot 10^4}{91}$
δ-Tocopherol	Methanol	298	87	–	$\frac{A_{298}\cdot 10^4}{87}$
Retinol	Ethanol (2-propanol for QA)	325	1830	$A_{300nm}/A_{325\ nm} \leq 0.602$ $A_{350nm}/A_{325\ nm} \leq 0.452$ $A_{370nm}/A_{325\ nm} \leq 0.093$	$\frac{A_{325}\cdot 10^4}{1830}$
Lutein	Ethanol	445	2550	–	$\frac{A_{445}\cdot 10^4}{2550}$
β-Carotene	Hexane	453	2592	$A_{455nm}/A_{340\ nm} \leq 15$ $1.14 \leq A_{455nm}/A_{483\ nm} \leq 1.18$	$\frac{A_{435}\cdot 10^4}{2592}$

[1] Absorbance.

acquired from Panreac Applichem (Castellar del Vallès, Barcelona, Spain). All solvents for chromatography: acetonitrile (99.9% purity), methanol (99.9% purity) and dichloromethane (99.8% purity) were HPLC gradient and obtained from Chem-Lab (Zedelgem, Belgium). In the case of standards, α-tocopherol (99.9% purity), γ-tocopherol (97% purity), retinol (97.5% purity), cholesterol (99% purity), lutein (97% purity) and β-carotene (97% purity) were purchased from Sigma-Aldrich (Sant Louis, Missouri, USA).

2.2. Standard preparation

2.2.1. Stock solution preparation

The vitamins and carotenoids standards were dissolved in their corresponding solvent (Table 1) to obtain solutions of 50 μg ml^{-1} for tocopherols and 2.5 μg ml^{-1} for retinol, lutein and β-carotene. Cholesterol was dissolved in acetonitrile:dichloromethane:methanol (75:10:15, v:v:v) to obtain 3 mg ml^{-1} solution.

2.2.2. UV standardization of stock solutions

The integrity and the exact concentration of vitamins and carotenoids stock solutions were determined by spectrophotometry (Thermo Electron Corporation Heλios β spectrophotometer) under the conditions given in Table 1, following the indications found in applied bibliography (AENOR, 2014a,b,c; Chauveau-Duriot et al., 2010).

These stock solutions were stored at −80 °C in total darkness under nitrogen atmosphere.

2.2.3. Working standard solution preparation

Each standard solution was diluted in acetonitrile:dichloromethane:methanol (75:10:15, v:v:v) and injected alone, to identify their retention time and spectra. Then, five-point calibration curves were built from a mix of all standard solutions prepared in the same mixture of solvents; 20 ng ml^{-1}–5000 ng ml^{-1} for tocopherols, 4 ng ml^{-1}–400 ng ml^{-1} for retinol, 20 ng ml^{-1}–400 ng ml^{-1} for lutein and β-carotene and 20 μg ml^{-1}–1500 μg ml^{-1} for cholesterol respectively.

2.3. Samples

2.3.1. Sampling

The study was performed using samples of milk and meat of *Longissimus dorsi* muscle, liver, subcutaneous caudal fat and perirenal fat of suckling lambs from an experiment conducted in CITA research station (for details Lobón, Sanz, Blanco, Ripoll, and Joy (2017)). Briefly, 20 pairs of ewe-suckling lamb grazed in mountain pastures until lambs reached target slaughter weight 10–12 kg. Milk and meat samples were protected from direct light with aluminium foil and frozen. The samples of subcutaneous caudal fat, perirenal fat, liver, and *Longissimus dorsi* muscle were obtained *post-mortem* after cooling at 4 °C for 24 h. The samples were lyophilised, except for fat samples, in a Genesis Freeze Dryer 25 (Hucoa Erlöss, SA/Thermo Fisher Scientific, Madrid, Spain) with chamber temperature: −20 °C, chamber pressure: 113 mTorr, condenser temperature: −80 °C, subsequently stored in vacuum-opaque packages and frozen at a temperature of −80 °C. A sample of each matrix of a ewe-lamb pair, selected randomly, was analysed in quintuplicate.

2.3.2. Samples treatment

The method of extraction of the analytes was the same for all the animal matrices, differing only in the quantity of sample used: 100 mg of lyophilised milk, 200 mg of lyophilised meat, 50 mg of lyophilised liver and 100 mg of fat. Each sample was placed in a 15 ml polypropylene centrifuge tube. Following the indications of Prates et al. (2006), 200 mg of L-ascorbic acid and 3 ml of saponification solution (10% w/v potassium hydroxide in ethanol:distilled water 50:50 v:v mixture) were added. The mixture was vortexed quickly under nitrogen atmosphere. The saponification procedure was performed in an orbital shaker (600 rpm) at room temperature (23 °C approximately) overnight.

Then, 5 ml of *n*-hexane:ethyl acetate 9:1 v:v with 5 μg ml^{-1} of BHT mixture were added (AENOR, 2014a,b,c). The tubes were vortexed for 10 s, shacked in a an orbital shaker (600 rpm) for 15 min and centrifuged at 2000 × *g* at 10 °C for 5 min. The upper layer (organic solution) was recovered in a 12 ml amber glass tube. This procedure was repeated twice. The organic solution was evaporated in a rotational vacuum concentrator at 40 °C for 30 min. Then, the residue was dissolved in 1 ml of mobile phase acetonitrile:methanol:dichlorometane (75:15:10, v:v:v), shacked in an orbital shaker (600 rpm) for 10 min at room temperature and filtered through a 0.2 μm–13 mm PTFE syringe filter into a 2 ml amber screw-cap vial for UHPLC.

2.4. UHPLC conditions and methods

The chromatographic system is an ACQUITY UPLC H-Class liquid chromatograph (Waters, Milford, Massachusetts, USA) equipped with a silica-based bonded phase column (Acquity UPLC HSS T3, 1.8 μm × 2.1 mm × 150 mm column, Waters) an absorbance detector (Acquity UPLC Photodiode Array PDA eλ Detector, Waters) and a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters). The UHPLC system was controlled by the Empower 3 software.

The mobile phase was acetonitrile:methanol:dichlorometane (75:15:10, v:v:v) with a flow rate of 0.3 ml min^{-1}. The temperature of the samples and the column were adjusted to 25 °C and 35 °C, respectively. The injection volume was 5 μl and the total run time of the chromatographic procedure was 15 min (20 min if β-carotene was present).

Tocopherols were detected by fluorescence emission at 295 excitation wavelength (λ_{exc}) and 330 nm emission wavelength (λ_{emi}), retinol and carotenoids (lutein and β-carotene) by absorbance at 325 nm and 450 nm, respectively; and cholesterol at 220 nm. The analytes in the different matrices were identified by comparison of the retention times and spectral analysis with those of the pure standards.

2.5. Statistical analysis

For each analyte, the sensitivity was determined as the slope of the calibration curve. The limits of detection and quantification were set as 3 or 10 times the standard deviation of ten blanks (5 μl of mobile phase)