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RED YEAST β-CAROTENE CONTENT: DEVELOPING EXTRACTION AND DETERMINATION FOR IMPROVING POULTRY NUTRITION

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Abstract

Vitamin A is an essential nutrient, for both production and reproduction of farm animals, however, most animals are unable to synthesize de novo precursors of vitamin A (β-carotene), and dietary supplementation is mandatory. The current paper aims to evaluate the analytical parameters and to improve the method efficiency for determining β-carotene content in red yeast and the internal laboratory validation protocol. Our method stands for the ultraviolet-visible spectrophotometric uses, having the Lambert-Beer law as the basis. To determine the β-carotene content, dimethyl-sulfoxide was employed as a solvent, calibration curve, and visible spectra were evaluated (300-900 nm). The linearity of beta carotene measured at 465 nm using the UV-Vis method linearity range was 6.1-36.6 µg/ml, R^2=0.997, LOD = 5.39 µg/ml, LOQ = 16.32 µg/ml, SD less than 5% and RSD in between 1-15%. In conclusion, the β-carotene spectroscopic method determination is a cheap, and efficient method, suitable for β-carotene determination and retinol and retinoic acid estimation for nonconventional feed additives such as yeasts.

Key words*: β-carotene, retinol, spectrophotometry, UV-Vis method.*

INTRODUCTION

Red yeasts can synthesize carotenoid complex (Frengova & Beshkova, 2009) and might be an alternative to synthetic vitamin and pigment dietary additives used in livestock (Marounek & Pebriansyah, 2018; Meléndez-Martínez et al., 2022). Recent interest in the utilization of microbial β-carotene sources has been stimulated by reproductive performance in poultry (Sajjad et al., 2020), but also by the meat and egg quality improvement when the dietary supplementation took place (Kanwugu et al., 2021; Sun et al., 2020). Poultry farming is an important branch of agriculture in many countries and the main challenge is driven by the organic farms producing units, unable to utilize chemical additives (Pailliè-Jiménez et al., 2020; Pandey & Kumar, 2021), and organic diets are mandatory. In poultry physiology, carotenoid synthesis is absent and the exogenous dietary intake is essential (Ortiz et al., 2021), for further conversion of both xanthophylls and retinal/retinoic acid. Common farming practice employing chemically developed formulas of carotenoids as pigment additives (Ribeiro et al., 2018), for example, β-Apo-8′-carotenal, or canthaxanthin (Surai & Kochish, 2020) is often a conventional dietary strategy for both broiler chicks (Grashorn, 2016) and laying hens (Ortiz et al., 2021), in order to supplement the nutritional requirements (broilers - 10000 IU vitamin A, and laying hens 6500 IU vitamin A) for improving product quality attributes (ISA, 2020; NRC, 1994).

The study of the chemical composition, and the determination of the complex of carotenoids, in the matrix of non-conventional raw materials, is an important step in their potential nutritional use. Considering the fact that the complex carotenoid matrix presents in yeast such as *Rhodotorula mucilaginosa* is directly influenced by the strain genetic determinism (Pino-Maureira et al., 2021) and productivity also modulated by both yeast phenotype (Zhang et al., 2016) and the engineering approach via metabolites enhancement (Verma et al., 2019). Furthermore, adapting and improving the in-processes followed by

quantitation and qualifications of productivity to obtain product maximization with the minimal economic implication is a novel trend.
UV-Vis (UV-visible spectrophotometry) is a spectroscopic approach, absorption or reflection spectroscopy in the ultraviolet part and the complete adjacent regions of the electromagnetic spectrum (Popescu et al., 2022). It is a relatively cheap and easy-to-use instrumental application, also this methodology is widely used in various fundamental connected domains. Absorption spectroscopy is complementary to fluorescence spectroscopy.
The paper focuses on the chemical instrumental method extraction and development of yeasts carotenoids, while improving the dietary carotenoid dosage retinoic acid estimation for poultry nutrition.

MATERIALS AND METHODS

The method development basis was previously described by (Barba et al., 2006), employing hexane and acetone as extractive complex solvents.
In order to develop the instrumental UV-Vis method of yeasts carotenoids, the following reagent, and reference standard were used: dimethyl sulfoxide (DMSO, BioReagent, suitable for hybridoma, ≥99.7%, Sigma-Aldrich, Missouri, USA), type I β-carotene reference standard (synthetic, ≥93%, suitable for UV determination, powder Sigma-Aldrich, Missouri, USA), ultrapure water (chromatographic resistivity at least 18,4 MΩ, and having the total organic substances max. 29 ppb). Previously, three batches of *Rhodotorula mucilaginosa* biomass were optimized (nutritive substrate, on an orbital shaker New Brunswick Scientific, Innova40®, Germany) for biomass yield and biomass carotenoid complex (unpublished data). For the carotenoid extraction, the yeast biomass was previously lyophilized at -50 °C, at 0.370 mBar (Labconco freeze dryer, Kansas, USA) and weighed on an analytic balance (Precisa XT220A, Switzerland).

Analyte extraction
The 96-h fermented *Rhodotorula mucilagionosa* lyophilized biomasses (3 replicas/batch) were individually resuspended in 2.5 ml of DMSO, ultrasonicated for 15 minutes (Elmasonic S 50 R, Germany), and centrifugated at 4°C, 10000 rpm, during 5 minutes (Sigma 2-16PK refrigerant centrifuge, Germany). The collected yeast pellets were washed (as mentioned before), during four successive cycles, finally collecting the amount of 10 ml supernatant/per each sample washed.

Reference standard preparation
The β-carotene stock solution was prepared, by solubilization in DMSO, having a concentration equal to 366.66µg/ml. The working standard solution was prepared freshly, in DMSO, and spiked (6.1, 9.15, 12.2, 15.5, 18.3, 21.35, 24.4, and 36.6 µg/ml), for further efficacy, a standard etalon curve was developed.
In order to determine the analytical recovery and method precision, the amount of β-carotene present in each yeast's samples were calculated, using the linear regression equation, after developing the standard curves, just by taking into consideration the absorbance (optical density, OD values). Triplicate determinations were employed for each sample, and at the end was calculated the percentage of the recovery. For all three yeast batches samples, were calculated the standard deviations (SD) and the relative standard deviations (RSD), by analysing the minimum, maximum, and average concentrations, three sets for each experimental batch, when compared with the blank.

Selectivity
The ability of a method to identify particular analyte) in a complicated mixture without becoming interfered with by other mixture constituents is referred to as selectivity. Specific means that a procedure is completely selective for a given analyte or group of analytes (Verbić et al., 2013).
For determining the method specificity, chemical (NaOH 0.1N and HCl 1M), and physical-chemical (heat stress - exposed during 2h, and 3h at 40°C) processes were employed, for influencing the analyte structure in the reference solution (36.6 µg/ml) and product tests solutions (5.22, 5.31, and 4.73 µg/ml), in triplicate. RS solution: 3.66 mg of standard reference was diluted to a balloon of 10 ml with

DMSO. TS solutions: 10 ml of supernatant collected after washing the yeast pellets with DMSO. Blank: 10 ml DMSO. Stress 1: 2 ml of TS, and 0.5 ml of 0.1N NaOH, diluted to balloon 10 ml, with DMSO. Stress 2: 2 ml of TS, and 0.5 ml of 1M HCl, diluted to balloon 10 ml, with DMSO. Stress 3: 2 ml of TS, diluted to 10 ml DMSO, and exposed for 2h at 40°C drying oven (Binder, ROTH, Germany). Stress 4: 2 ml of TS, diluted to 10 ml DMSO, and exposed for 3h at 40°C drying oven (Binder, ROTH, Germany).
Each stress test solution (n = 24) spectra were evaluated for maximum absorbance read, and overlaying characteristics.

Linearity
The capacity of an analytical process to produce test results that are directly proportional to the concentration (quantity) of analyte in the sample is known as linearity (Guy, 2014). The linear concentration range and the assay variability, mandatory imply the development of the limit of detection and the limit of quantification. The lowest quantity or concentration of a component that can be consistently detected using a particular analytical procedure is commonly referred to as the limit of detection (LOD). The smallest amount or lowest concentration of a material that can be determined using a certain analytical process with the established accuracy, precision, and uncertainty is referred to as the limit of quantification, or LOQ.

$$\mathrm{LOD} = \frac{3.3*\sigma}{S},$$
$$\mathrm{LOQ} = \frac{10*\sigma}{S},$$

having the σ – the standard deviation of the response, and S – the slope of the calibration curve. Stock solution: RS solution: 3.66 mg of standard reference was diluted to a balloon of 10 ml with DMSO. Linearity solution 6.1 ppm: 50µl TS to 3 ml DMSO. Linearity solution 9.2 ppm: 75µl TS to 3 ml DMSO. Linearity solution 12.2 ppm: 100 µl to 3 ml DMSO. Linearity solution 18.3 ppm: 150 µl to 3 ml DMSO. Linearity solution 36.6 ppm: 300 µl to 3 ml DMSO. Reading in triplicate each linearity solution, calculating the β-carotene mean absorption value, SD standard deviation (%), and RSD (%) relative standard deviation.

Precision
The degree of agreement among individual test findings when the method is performed on several samplings of a homogenous sample is defined as the precision of an analytical method. The standard deviation or relative standard deviation (coefficient of variation) of a sequence of measurements is commonly used to express the precision of an analytical technique (Guy, 2014). Often, the method precision attribute is known as repeatability. For this assay, two analysts were employed, preparing a similar sample solution for determining the β-carotene content present in the 36.6 ppm solution. Sample preparation: 36.6 ppm β-carotene in DMSO. The simple solutions were evaluated six-time, saving the maximum value of absorption, wavelength (465nm) for calculating the mean values, the SD, and the RSD (%) ≤ 5%.
Statistical data
All data were analysed using the XLSTAT for Excel 2021 version software (Addinsoft, New York, USA).

RESULTS AND DISCUSSIONS

Selectivity
Stress solutions and reference standard solutions spectra (300-900 nm) were developed by using the quartz 1cm cuvettes (Figure 1). The percentage of the analyte recovery is displayed in Table 1. The lower results were obtained when the red yeast sample solution was exposed to heat, for 2 h (82.32 ± 3.45), and 3 h (74.56 ± 3.57), when compared with the reference solution. Similar to our findings, suggests that heat influenced the β – carotene present in food (Borba et al., 2019) and feed (Thakur, 2018).

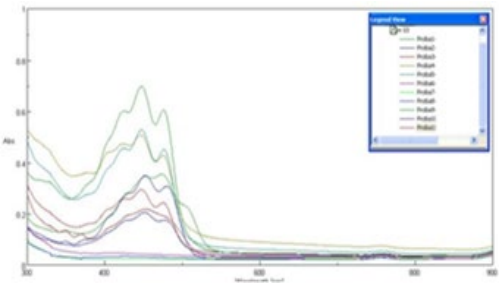

Figure 1. β-carotene in DMSO method selectivity

Our method possesses the selectivity attribute, thus the analyte effective separation. At the same time, no peaks were detected, indicating that our stress solutions are not interfering with the β-carotene assay, and could not influence the determination. Furthermore, the acceptance criteria implied the efficient separation of β-carotene from the red yeast matrix, under different conditions. The maximum absorption was at 465 nm, specific for the β-carotene standard reference (Chábera et al., 2009).

Table 1. Stress solution β-carotene content variations

Analyte recovery	0.1N NaOH	1M HCl	2h40°	3h40°
Mean	97.64	101.5	82.32%	74.56
SD	3.42	2.38	3.45	3.57
RSD	2.76	3.23	3.88	4.22

Linearity

A linear regression graphic was developed, having the general equation y = ax + b, the abscissa originating 0, indicating the linear solution concentration and the ordinate axis, resulting in the linear solution absorption. For validating the instrumental method linearity, the regression coefficient ($R^2 \geq 0.997$) was calculated, having the RSD %, less than 5%.

In Table 2 and Figures 2 and 3 the method's linearity assay results are displayed.

Table 2. Test solutions recovery percentage

Test solution	Mean value	SD	RSD (%)
6.10 ppm	0.0479	0.00090185	1.88015973
9.15 ppm	0.0669	0.00065064	0.98185217
12.20 ppm	0.0841	0.00110942	1.30087467
18.30 ppm	0.1090	0.00041633	0.38242486
36.60 ppm	0.1280	0.00100000	0.78740157

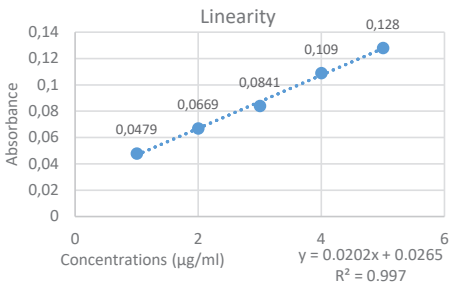

Figure 2. β-carotene linear range

Our results indicate that the studied concentration range (6.1-36.6 ppm) is linear, with 99.7% of accuracy, and the LOD and LOQ values are corresponding to the EPh. requirements (Bouin & Wierer, 2014), having LOD's signal-to-noise ratio, S/N≥3, and LOQ's ratio S/N≥10. The RSD is less than 5%, and the R2 is higher than 99.7% for the β-carotene assay.

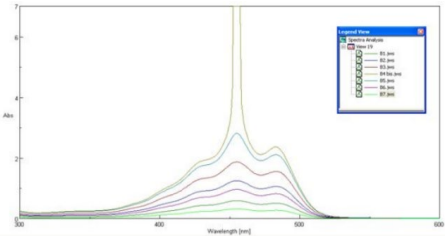

Figure 3. Linearity range of β-carotene in DMSO

Precision

The method repeatability results are presented in Table 3 and Figure 4. We assume that the within-samples standard deviation is the same throughout the range, in order to estimate it we evaluate both, in between the samples (intra-individual variability) and in between the analyst's repeated measurements (inter-observer variability), the maximum RSD, often regarded as the closeness of agreement was 1.88%; Karnjanawipagul P., and W. Nittayanuntawech, 2010).

Table 3. β-carotene repeatability and reproducibility

Sample	Analyst 1			Analyst 2		
1	0.1278	0.1282	0.1286	0.1284	0.1280	0.1278
2	0.1264	0.1262	0.1267	0.1265	0.1262	0.1273
3	0.1279	0.1274	0.1274	0.1272	0.1268	0.1281
Mean	0.1274	0.1273	0.1276	0.1274	0.1270	0.1277
SD	0.0008	0.0010	0.0010	0.0010	0.0009	0.0004
RSD	0.6585	0.7910	0.7533	0.7544	0.7217	0.3164

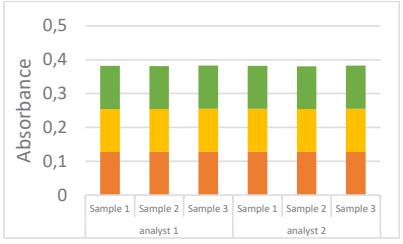

Figure 4. Repeatability and reproducibility of β-carotene in DMSO

CONCLUSIONS

The spectrophotometric detection of β-carotene from red yeasts was successfully developed having the DMSO as an extractive solvent. The current method met both the efficiency and speed tests for three batches of red yeast pigments. Additionally, simultaneous assays were accomplished, having high scores of repeatability and reproducibility. This instrumental method is a cheap, and efficient, suitable for β-carotene determination and retinol and retinoic acid estimation from conventional and nonconventional poultry feed additives such as yeast. Furthermore, the current method presents a great potential for the rapid pigment evaluation and might be suitable for broilers serum blood β-carotene determination.

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