Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1025 (2004) 313–317
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

Simultaneous determination of Vitamin E homologs in chicken meat by liquid chromatography with fluorescence detection

A.K. Hewavitharana [a,*], M.C. Lanari [a], C. Becu [b]

[a] *CSIRO, Food Science Australia, Cannon Hill, Qld. 4170, Australia*
[b] *Ecole Nationale Supérieure de Biologie Apliquee a la Nutrition et a la Alimentation (ENSBANA), Dijon, France*

Received 17 July 2003; received in revised form 7 October 2003; accepted 16 October 2003

Abstract

A simple and reliable method for the simultaneous determination of all eight homologs of Vitamin E in chicken meat is described. All analytes, including the internal standard (α-tocopherol acetate), were eluted within 35 min and detected using their native fluorescence (295 nm excitation and 330 nm emission). Chromatography using hexane based eluent on a normal phase silica column included an initial column conditioning step to prevent irreversible adsorption of tocopherols and tocotrienols on silica. Lowest detectable levels of α-tocopherol, γ-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol were 0.73, 0.86, 1.0, 1.2, 1.7 and 1.3 ng, respectively.

Keywords: Meat; Food analysis; Vitamins; Tocopherols; Tocotrienols

1. Introduction

Vitamin E plays a fundamental role in the prevention of radical formation in biological systems like plasma, membranes and tissues. Vitamin E is the collective name for the eight naturally occurring forms α, β, γ and δ tocopherols and α, β, γ and δ tocotrienols. Historically, α-tocopherol was reported to have the highest biological activity [1] thus most methods available for Vitamin E assay were developed exclusively for the determination of α-tocopherol. However, many studies have demonstrated that the antioxidant activities of β, γ and δ tocopherols were also important [2]. Yamaoka et al. [3] showed that α-tocotrienol had a higher antioxidant activity than α-tocopherol in a phopholipid liposome solution. Dietary tocotrienols reduced plasma lipid peroxidation in humans [4], and α-tocotrienol decreased the susceptibility of low-density lipoptoteins (LDLs) to copper induced oxidation [5].

The use of plant extracts rich in tocopherols and tocotrienols in the pharmaceutical and food industries is becoming increasingly popular, generating a great need for a fast and efficient technique for separating and quantifying the individual Vitamin E analogs. Although several methods have been developed to determine other forms of Vitamin E in various matrices [2], no methods were reported for the separation and/or quantification of all eight vitamin analogs in animal muscle The aim of the present study was to develop a technique for measuring the composition of tocopherols and tocotrienols in meat from chickens supplemented with extracts rich in these antioxidants.

In this study, we selected an extraction method that claimed to exhibit the minimum loss of Vitamin E [6] which did not include saponification, then further modified to minimize oxidative losses. Precautions were taken in order to prevent inaccuracies in quantification due to irreversible adsorption of analytes on to the silica column, and fluorescence quenching of tocopherol signal at the detection step of chromatographic analysis. The accuracy, precision and robustness of the method were improved by incorporating an internal standard from the extraction step through to the chromatographic analysis. As well, the use of fluorescence rather than UV as the detection mode provided the sensitivity and the selectivity required for the accurate determination of low levels of these homologs in muscle tissue.

* Corresponding author. Present address: Investigative Chemistry, Queensland Health Scientific Services, P.O. Box 594, Archerfield, Qld 4108, Australia. Fax: +61-7-3274-9123.
E-mail address: Amitha_Hewavitharana@health.qld.gov.au (A.K. Hewavitharana).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.10.052

2. Experimental

2.1. Instrumentation

Chromatography was performed using Waters Associates chromatographic equipment (Milford, MA, USA) consisting of a 501 pump, a model 717 plus auto sampler, a model 470 scanning fluorescence detector, a pump control module, a SAT/IN module, and the program Millenium[32] (version 3.05.01). A Lichrosphere Si 100 silica column (5 μm, 250 mm × 4.6 mm) was used for all separations.

2.2. Reagents

α-Tocopherol, α-tocopherol acetate, γ-tocopherol, butylated hydroxytoluene (BHT), and 1,4-dioxane were purchased from Sigma (St. Louis, MO, USA), and the tocotrienol standards were from Calbiochem (San Diego, CA, USA). Rice oil extract was from Eastman (Kingsport, TN, USA). Dioxane, acetic acid and ethanol were all analytical reagents from Ajax Chemicals (Sydney, Australia). Double deionized water was obtained from an IBC water purification system (Mansfield, Qld, Australia), and HPLC grade hexane was from Mallinckrodt (Paris, KY, USA).

2.3. Mobile phase

Mobile phase consisted of 4% 1,4-dioxane, 0.04% acetic acid and 0.02 mg/l α-tocopherol in hexane. It was filtered through a PTFE 0.45 μm filter (Millipore C, Bedford, MA) and degassed before each HPLC session.

2.4. Standard solutions

Stock standard solutions of α-tocopherol (2000 mg/l), α-tocopherol acetate (2000 mg/l), γ-tocopherol (1000 mg/l), and each of the tocotrienols (2000 mg/l) were prepared in hexane. A combination standard containing α-tocopherol (10 mg/l), α-tocopherol acetate (75 mg/l), γ-tocopherol (5 mg/l), α-tocotrienol (2 mg/l), β-tocotrienol (2 mg/l), γ-tocotrienol (5 mg/l), and δ-tocotrienol (2 mg/l) was prepared by diluting appropriate volumes of stock solutions with hexane containing 200 mg/l BHT, and used for the quantification of muscle samples. The internal standard solution added to each muscle sample was prepared by diluting α-tocopherol acetate stock solution with hexane to 200 mg/l. All of the above solutions were frozen at −20 °C. The combination standard was frozen as small aliquots, and thawed daily as required.

2.5. Sample preparation

Chickens were supplemented with extracts rich in tocopherol and tocotrienols for 42 days prior to slaughter. The breast (*Pectoralis M.*) was removed at 24 h postmortem, vacuum-packed and stored at −20 °C until required for analysis.

A portion of each breast was minced, and a representative sample of 1 g was placed in a 50 ml polyethylene tube. A 300 μl aliquot of 200 mg/l α-tocopherol acetate solution (internal standard), and 4 ml of absolute ethanol were added. The mixture was homogenized for 30 s in a UltraTurax T25 (Janke et Hunkel IKA-Labortechnik, Kuala Lumpur, Malaysia) homogenizer with a 18 G dispersing element. Five milliliters of distilled water were added to the tube and the content homogenized for 15 s. A 4 ml aliquot of hexane containing 200 mg/l BHT was pipetted and the sample homogenized for further 15 s. The tubes were capped and centrifuged at 1500 rpm for 10 min. A 100 μl injection volume of the upper (hexane) layer was used for HPLC analysis.

2.6. Chromatographic conditions

Chromatography was performed at ambient temperature. Flow rate used was 1 ml/min. The column was equilibrated for 15 min with the mobile phase prior to injection of each sample or standard. All samples were detected using fluorescence at 295 nm excitation and 330 nm emission.

2.7. Qualitative and quantitative analysis

Qualitative analysis was performed by spiking the sample with appropriate standard to observe the growth of the peak, and by comparing the order of elution with that reported in other studies.

Quantitative analysis was performed by injecting a range of volumes of the combination standard (in order to cover the expected concentrations of analytes in the extracts) with each batch of samples analyzed on the HPLC. The concentrations of the analytes in the samples were determined using a 4-level calibration curve constructed from the standard data. Quantification was performed by the internal standard method to compensate for the sample losses during extraction and chromatographic analysis.

3. Results and discussion

Ruperez et al. [2] compared reversed- and normal-phase liquid chromatography for the separation of various tocopherols and tocotrienols, and concluded that normal-phase liquid chromatography provided a superior resolution of these compounds. The authors [2] concluded that the reversed phase was not capable of separating β and γ tocopherols. Normal-phase liquid chromatography using silica is well known for its unique capability of discriminating isomers [7]. As normal-phase liquid chromatography is performed with non-polar organic solvents, it also presents the advantage of high solubility for lipids [2], and therefore lipid soluble vitamins such as Vitamin E. Kamal-Eldin et al. [8] compared different types of normal phases for the separation

of vitamin isomers and found that silica columns performed the best.

In this study, we used isopropanol, ethanol and 1,4-dioxane as modifiers in a hexane mobile phase and found that 4% 1,4-dioxane produced the best resolution of all analyte peaks, which is consistent with the findings by Kamal-Eldin et al. [8]. In addition, we added 0.04% acetic acid to reduce peak tailing [9], and 0.02 mg/l α-tocopherol to prevent analyte loss due to irreversible adsorption.

We observed that the first few injections of the standards in each HPLC session have produced either no or small peaks and that these peaks produced deviation from linearity in the calibration curve. Addition of 0.02 mg/l α-tocopherol to the mobile phase and injection and elution of two 200 μl aliquots of 2000 mg/l α-tocopherol eliminated this problem. We hypothesized that tocopherols and tocotrienols were irreversibly adsorbed on the silica column. Therefore, blocking these sites with tocopherols/and or tocotrienols before running the samples restores the normal chromatographic peak areas [10]. The amount of tocopherol required to block the extra active sites will vary from column to column. The procedure can be repeated, however, until consistent peak areas are obtained for the analyte of interest.

Katsanidis and Addis [6] compared different extraction methods for Vitamin E in bovine muscle and found that a non-saponification approach gave the best recovery. This approach presented an additional advantage in that, unlike saponification, it does not convert α-tocopherol acetate to α-tocopherol, and so enabled us to use α-tocopherol acetate as the internal standard. The extraction method used in the current study was a modified version of this non-saponification approach incorporating α-tocopherol acetate (as the internal standard), and BHT to the extracting solvent (hexane) to protect analytes from oxidation.

α-Tocopherol acetate was used as the internal standard as it closely resembles the major Vitamin E homolog (α-tocopherol) in muscle. Contrary to the claims that it has no fluorescent properties [2], we found that it fluoresced

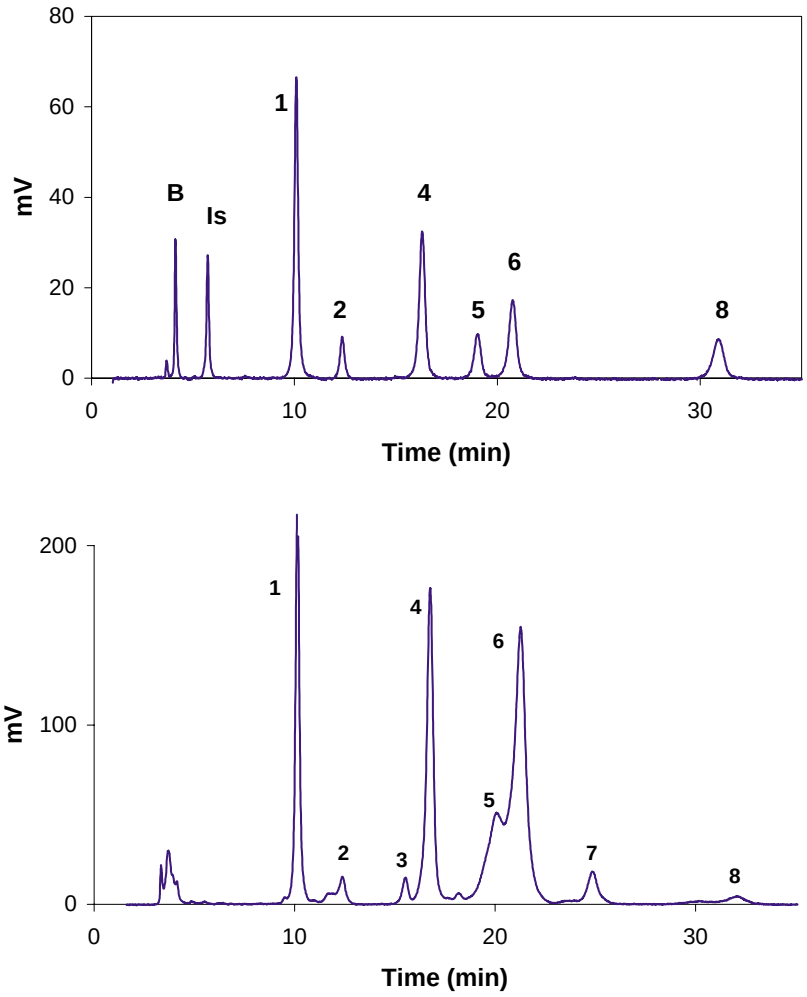

Fig. 1. Chromatogram of a combined standard solution (upper diagram) and chromatogram of a rice oil extract (lower diagram). Peaks: B, BHT; Is, internal standard/α-tocopherol acetate; 1, α-tocopherol; 2, α-tocotrienol; 3, β-tocopherol; 4, γ-tocopherol; 5, β-tocotrienol; 6, γ-tocotrienol; 7, δ-tocopherol; 8, δ-tocotrienol.

adequately for detection as an internal standard. As its signal was weaker than other tocopherols or tocotrienols, relatively larger amounts of α-tocopherol acetate were added to both the combination standard and the samples in order to obtain comparable peak sizes. BHT and α-tocopherol acetate produced chromatographic peaks that were well separated from the other sample peaks. Blank runs of the muscle extracts showed that BHT and α-tocopherol acetate were absent in muscle samples. We also confirmed from the blank runs that the α-tocopherol acetate (internal standard) peak in particular was clear from any co-eluting substances in the samples, ensuring high accuracy in the quantification step.

Another precaution to be taken in the analysis of Vitamin E is that, when fluorescence is used as the mode of detection, the peak size diminishes with time if the mobile phase is not adequately degassed. This reduction in fluorescence was attributed to the quenching of the fluorescence signal by oxygen [11].

Fig. 1 (upper diagram) shows the chromatogram for the combined standard. Excellent selectivity and sensitivity were achieved for six of the eight homologs present in the combined standard, α-tocopherol acetate, and BHT. As standards for β-tocopherol and δ-tocopherol were not available, a commercial rice oil extract (50 mg/l solution in hexane) containing all Vitamin E homologs was chromatographed in order to test the ability of our chromatographic method to separate all eight homologs, and the results shown in Fig. 1 (lower diagram). Peaks were identified by spiking the rice oil extract with individual standards before been chromatographed, and by comparison of the order of elution obtained for standards in other studies using similar conditions for elution [8]. Clearly, the method was capable of separating all eight homologs.

A typical chromatogram obtained for chicken breast muscle is shown in Fig. 2. Only two homologs of Vitamin E were present in the samples used in our experiment, α- and γ-tocopherol. The other homologs were either absent or present below the limits of detection. The bottom diagram of Fig. 2 shows a chromatogram obtained for a muscle extract spiked with α-tocopherol, γ-tocopherol and all

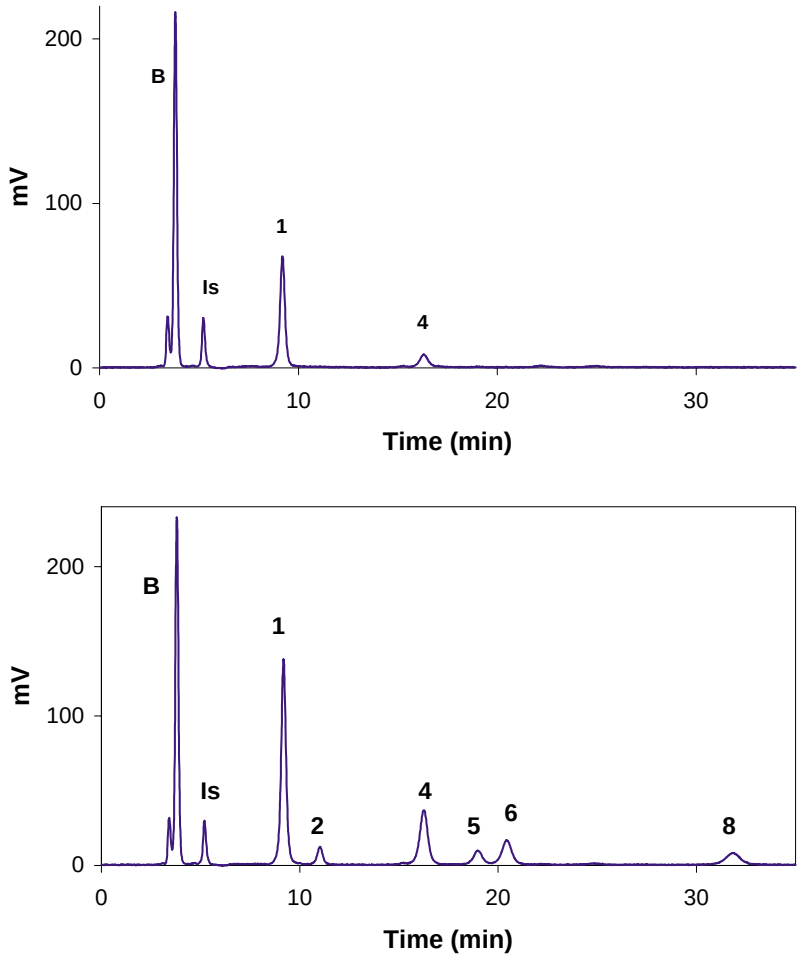

Fig. 2. Chromatogram of a typical chicken meat extract (upper diagram) and chromatogram of a chicken meat extract spiked with two tocopherols and four tocotrienols (lower diagram). Peaks: B, BHT; Is, internal standard/α-tocopherol acetate; 1, α-tocopherol; 2, α-tocotrienol; 4, γ-tocopherol; 5, β-tocotrienol; 6, γ-tocotrienol; 8, δ-tocotrienol.

four tocotrienols, demonstrating the ability of the method to separate and quantify all homologs if they were present in chicken meat. As there were only two homologs present in our chicken meat samples, recovery studies were performed only on these homologs. Meat samples were spiked with standard compounds of these two homologs, then subjected to the extraction procedure and HPLC separation. The recovery percentages obtained for α- and γ-tocopherol were 98 and 93%, respectively. Calibration curves were linear ($R^2 > 0.99$) at least up to 0.5, 0.25 and 0.1 μg for α-tocopherol, γ-tocopherol, and the four tocotrienols, respectively. Detection limits were estimated at a signal to noise ratio of 3:1, which correspond to 0.73, 0.86, 1.0, 1.2, 1.7 and 1.3 ng for α-tocopherol, γ-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol, respectively.

4. Conclusion

A simple, robust method was developed for the simultaneous determination of tocopherols and tocotrienols in chicken meat using α-tocopherol acetate as an internal standard. The importance of column conditioning and mobile phase degassing were emphasized in obtaining consistent, accurate results for all analytes of interest. This method will assist scientists interested in obtaining accurate values for all individual tocopherols and tocotrienols in chicken meat, and possibly in other animal tissues.

Acknowledgements

The assistance of Ms. Sharon Rablin and Mr. Ron Kuypers is gratefully acknowledged. This research was made possible by funding from the Commonwealth Scientific and Industrial Research Organisation (CSIRO). C.B. was funded by the Conseil Regional de Bourgogne (France).

References

[1] J. Bunyan, D. McHale, J. Green, S. Marcinkiewicz, Br. J. Nutr. 15 (1961) 253.
[2] F.J. Ruperez, D. Martin, E. Herrera, C. Barbas, J. Chromatogr. A 935 (2001) 45.
[3] M. Yamaoka, M.J. Carrillo, T. Ankara, K. Komiyama, J. Am. Oil Chem. Soc. 68 (1991) 114.
[4] R.P. Mensink, A.C. van Howelingen, D. Kromhout, G. Hornstra, Am. J. Clin. Nutr. 69 (1999) 213.
[5] D. O'Byrne, S. Grundy, L. Packer, S. Devaraj, K. Baldenius, P. Hoppe, K. Kramer, I. Jialal, M.G. Traber, Free Radic. Biol. Med. 29 (2000) 834.
[6] E. Katsanidis, P.B. Addis, Free Radic. Biol. Med. 27 (1999) 1137.
[7] D.A. Skoog, Principles of Instrumental Analysis, CBS College Publishing, New York, NY, 1985, p. 817.
[8] A. Kamal-Eldin, S. Gorgen, J. Pettersson, A. Lampi, J. Chromatogr. A 881 (2000) 217.
[9] A.K. Hewavitharana, A.S. van Brakel, M. Harnett, Int. Dairt J. 6 (1996) 613.
[10] A.K. Hewavitharana, Anal. Biochem. 313 (2003) 342.
[11] A.S. van Brakel, A.R. Matheson, A.K. Hewavitharana, J. Chromatogr. Sci. 35 (1997) 545.