Protection of Carotenoids in Alfalfa Leaf Extracts by Ethoxyquin Addition and Cold Temperature Storage

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Synopsis


Alfalfa leaf extracts were prepared by disintegrating fresh herbage, coagulating the green juice by steam injection and separating the curd from the deproteinized juice. Addition of ethoxyquin (w/v) into the green juice at 0.01%, 0.02%, 0.05%, 0.10% and 0.20% levels prior to coagulation gave final concentrations of 0.11%, 0.26%, 0.70%, 1.15% and 2.57% in the freeze-dried leaf extract, respectively. Samples, packed in vinyl bags and wrapped in aluminum foils, were stored for 40 weeks at 28°C, 5°C or -18°C.

The lower the storage temperature, the lesser the reduction in carotenoid content was. The rate of loss of β-carotene was much faster than xanthophylls.

At high temperature (28°C), ethoxyquin was essential to reduce the loss of carotenoids. Without it, recoveries of β-carotene and xanthophyll in leaf extract were reduced to 3 and 15%, respectively, of the initial values within 40 weeks, but were improved to 30 and 50% by the addition of only 0.01% ethoxyquin. Further addition improved the recoveries a little further to 50 and 60%, respectively, at the highest application level.

At low temperatures (5 and -18°C), carotenoids were well preserved without ethoxyquin excepting β-carotene at 5°C. The recovery of β-carotene at 5°C was only 40% but was improved gradually to 90% with the increase of ethoxyquin levels.

The decline of ethoxyquin in leaf extract at temperature above freezing implies that ethoxyquin itself is subjected to degradation. Only slight difference was observed between 5°C and 28°C in the ethoxyquin degradation.

Key words: Alfalfa leaf extract, β-Carotene, Ethoxyquin, Storage temperature, Xanthophylls.

Introduction

Leaf extracts, formerly known as leaf protein concentrates (LPC) contain as much as 50-60% crude protein of high quality(16). They are also rich in carotenoids particularly xanthophyll which are essential in the poultry industry as sources of natural pigments for broiler's skin and egg yolks(10). In fact, the amount of xanthophyll present makes the leaf extract more important than the protein it contains(8). Thus, the stability of the carotenoids would add to its value.

The use of antioxidants(6) like ethoxyquin or reducing agents(9) has been proven to be effective in stabilizing the carotenoids in alfalfa meal(4), alfalfa leaf extracts(8,15) and poultry feeds(1). Ethoxyquin improves pigmentation not only by protecting the carotenoids in the feed per se but also by enhancing xanthophyll utilization during the digestion and absorption process(9).

Protection offered by storage at low temperature has also been reported(2,3,5,14). Carotenoid concentration decreased with rise in storage temperature. Maintaining storage chambers at very low temperature (such as freezers), however, entails huge cost of electricity and special facilities. In temperate countries, this cost may be reduced because of the winter season but in tropical regions this may not be afforded at all. Moreover, during transport or shipping this condition may not be adequately provided. Thus, it is necessary to determine the optimum temperature which would protect carotenoids at an affordable storage cost.

In our previous experiments, ethoxyquin excelled sodium metabisulfite, butylated hydroxyanisole,
ascorbic acid and β-tocopherol in reducing the loss of carotenoids in alfalfa leaf extract[12] but high level application of ethoxyquin detrimentally affected the performance and carotenoid accumulation of chickens consumed[11,12].

The objectives of the present experiment were (a) to identify the optimum level of ethoxyquin in the leaf extract that would protect the carotenoids, and (b) to determine the degree of protection ethoxyquin could provide at varying storage temperature.

Materials and Methods

Leaf extract preparation

Alfalfa (Medicago sativa L. cv. Natsuwakaba) was harvested at vegetative stage and processed according to the following stages: (i) disintegrating with a herbage crusher (Nihon Sharyo, Nagoya), (ii) pressing with a hydraulic press until pressure stabilized at 175 kpa, (iii) collection of the green juice and addition of antioxidant, (iv) steam precipitation of the green juice at 90°C, (v) collection of coagulum (leaf extract) in a continuous centrifuge, and (vi) freeze-drying of the leaf extract. The antioxidant used was Nonpurex (99.14% Ethoxyquin; 6-ethoxy-1,2-dihydro-2,4,6-trimethylquinoline), manufactured by Seiko Kagaku (Tokyo). It was added into the green juice (w/v) at the following levels: (a) 0.00% (control), (b) 0.01%, (c) 0.02%, (d) 0.05%, (e) 0.10% and (f) 0.20%.

About 5 g of leaf extract samples were packed into sealable vinyl bags and wrapped in aluminum foil. Before sealing, air was purged to ensure minimum oxygen level inside the packet. They were stored either at 28°C, 5°C or −18°C.

Chemical analysis

Sampling was done after 10, 20, 30 and 40 weeks of storage. Carotenoid content was determined by open column chromatography according to AOAC procedure[9]. The absorbance was read in a recording spectrophotometer (Shimadzu UV-160, Kyoto). Ethoxyquin content was measured by a spectrophotoflourometer (Shimadzu RF-1500, Kyoto) according to AOAC procedure[9].

Results

Fig. 1 shows the calibration curve and mathematical relationship between the amount of ethoxyquin added into the green juice during leaf extract preparation and the amount of ethoxyquin present in the dried leaf extract. The relationship was positive linear.

As shown in the footnotes of Figs 2 and 3, the average initial β-carotene and xanthophyll contents of leaf extracts treated with different levels of ethoxyquin were 1.070 and 1.609 mg/kg DM, respectivley.

The changes in the β-carotene and total xanthophyll contents at different storage temperatures are shown in Figs 2 and 3, respectively. At −18°C, both β-carotene and xanthophyll was preserved well for 20 weeks without ethoxyquin. Prolonging the storage would require to add 0.01 or 0.02% (w/v) ethoxyquin into green juice to protect both β-carotene and xanthophyll. At 5°C, the carotenoid loss in ethoxyquin-level dependent, particularly for β-carotene. At 28°C, ethoxyquin was very effective to preserve carotenoids even at the lowest level (0.01% of green juice) and without it, the loss was very rapid.

Fig. 4 shows the changes in the amount of ethoxyquin present in the leaf extract after 30 weeks of storage. It is evident that ethoxyquin itself was degraded at high temperature.

Discussion

A detail study on the yield of leaf extract from alfalfa had already been made[13,14] and it was about 120 g/kgDM at vegetative stage. Although the data are not shown in the text, similar results were obtained in the present study. The β-carotene and xanthophyll contents of the fresh leaf extracts shown in the footnotes of Figs 2 and 3, respectively, are 3 times as high as the values reported on freeze-dried alfalfa[5,10].

The effect of addition of ethoxyquin to leaf extract on the protection of carotenoids from the denaturation during storage had also been studied[15], but the applied level was so high that chicks fed a diet containing the leaf extract at 5% suffered detrimental effects[16]. The present study, therefore, was undertaken to find the critical application level of ethoxyquin to green juice for protecting carotenoids during storage at different temperatures.
Changes in δ-carotene content of leaf extracts stored for 40 weeks. The leaf extracts contained the following ethoxyquin concentration (mg/kg DM) before storage: ○ 1,150; ● 2,620; ▲ 7,000; □ 11,500; ■ 25,700. Mean initial δ-carotene content ± S.D. (mg/kg DM) was 1,070 ± 50.

Changes in the xanthophyll content of leaf extracts stored for 40 weeks. The leaf extracts contained the following ethoxyquin concentration (mg/kg DM) before storage: ○ 1,150; ● 2,620; ▲ 7,000; □ 11,500; ■ 25,700. Mean initial δ-carotene content ± S.D. (mg/kg DM) was 1,609 ± 119.

As shown in Fig. 1, the amount of ethoxyquin contained in the dried leaf extract was positively proportional to the level of ethoxyquin added into the green juice during processing. For a rough estimate, 1 g of ethoxyquin added per liter of green juice would give an approximate concentration of 1.2 to 1.3% in the freeze-dried leaf extract.

It is clearly shown in Figs 2 and 3 that both the carotenoids were decreased during storage at 28°C. The rate of decrease of δ-carotene was extremely rapid during the first 10 weeks of storage, particularly in the control but it slowed to a more steady rate later. After 10 weeks of storage, the control had only 11% and 42% of the initial δ-carotene and total xanthophyll content, respectively, while the leaf extract containing 1,150 mg ethoxyquin/kg DM retained 49% and 72%, respectively. Doubling each level of ethoxyquin gave only small differences. Storing at 5°C improved the stability and it was apparent that the resistance to deterioration was ethoxyquin.

Changes in the ethoxyquin content of leaf extracts after storing for 30 weeks.
level-dependent. The highest ethoxyquin content afforded the best protection. After 40 weeks, the \( \beta \)-carotene of the leaf extract containing 1,150 mg ethoxyquin/kg DM and stored at 5°C was similar to that containing 25 times as much ethoxyquin and stored at 28°C.

There was a similar trend in the decrease of the xanthophyll and \( \beta \)-carotene contents at 28°C. The rate of decrease, however, was slower in the xanthophyll than in \( \beta \)-carotene. This differential instability of \( \beta \)-carotene has been reported earlier\(^{4,11,13} \). The stability of xanthophyll at lower temperature is depicted at 5°C (Fig. 3B). Though the highest ethoxyquin level had the most stabilizing effect, as the storage was prolonged, ethoxyquin treatment at low concentration was essentially not different from the control.

The pattern of loss of both \( \beta \)-carotene and xanthophylls at 28°C and 5°C typified an autocatalytic radical reaction. GOLDMAN et al.\(^{6} \) demonstrated that \( \beta \)-carotene degradation curves are sigmoidal with 3 distinct regions; initiation, acceleration and retardation. The more rapid rate of deterioration of \( \beta \)-carotene and xanthophylls at 28°C than at 5°C may be associated with the accelerated activity of carotenoid degrading enzymes at higher temperature. Lipoxygenases are the major enzymes involved in carotenoid degradation. They are known to be thermostable and capable of forming reactive radicals. BEN AZIZ et al.\(^{13} \) pointed out that the concentration of lipoxygenase was linearly related to the bleaching of carotenoids. The xanthophyll degradation curve at 5°C suggests that the initiation or induction period was lengthened for about 10 weeks. The accelerated region was also not pronounced.

The behavior of both \( \beta \)-carotene and xanthophylls at freezing temperature (−18°C) is not easy to comprehend. There was no apparent decrease in \( \beta \)-carotene content with or without ethoxyquin until 40 weeks of storage except in the untreated leaf extract which started to decrease after 20 weeks. The \( \beta \)-carotene content in the control dropped by as much as 12% but ethoxyquin treated leaf extract, at any level, continued to have higher \( \beta \)-carotene than their initial contents. For xanthophylls, in the leaf extract containing 1,150 mg ethoxyquin/kg DM and in the control, the reduction after 40 weeks were 18 and 24%, respectively. For the other ethoxyquin treated leaf extracts, the trend is more difficult to assess but they appeared to have either the same or higher xanthophyll content than before storage. The higher values might be derived from some errors.

From the graph in Fig. 4, it is evident that ethoxyquin itself was degraded when temperature increased. After 30 weeks of storage, ethoxyquin content was reduced by as much as 60–75% and 35–60% at 28°C and 5°C, respectively. The reduced content of ethoxyquin could be possible reason for the continued decrease of carotenoids at these storage temperatures. But in situations where the leaf extract would be kept at high temperature, like during shipping or in tropical countries where there may not be enough good refrigeration/freezing systems, the use of antioxidant is essential. And the application level should be around 0.01% of green juice in view of animal health when leaf extract accounts for 5% of the diet\(^{17} \).

Though we were not able to identify which xanthophyll was most subject to changes in temperature and level of ethoxyquin, this study emphasized the proper use of ethoxyquin in preserving the carotenoids in leaf extracts stored at varying temperature.

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References


要 旨


アルファルファを収穫後ただちに破砕・圧搾して得た搾汁に0, 0.01, 0.02, 0.05, 0.1 及び 0.2% (w/v) のエトキシンを添加した。これらの搾汁を高圧蒸気により30℃に熱し、熟成後を処理機で分離し、乾燥乾燥したものと緑葉抽出物とした。各絞り抽出物のエトキシン含有率はそれぞれ0, 0.1, 0.2, 0.70, 1.15 及び 2.57% であった。これらをヒニールを入れ、さらにアルミパックで覆い、28, 5 及び -18℃の暗室中に4週間貯蔵した。貯蔵温度が低いかほどカロチノイドはよく保たれた。β-カロチノイドはキサンフォールよりも不安定であった。

高温（25℃）下における緑葉抽出物中のβ-カロチノイド及びキサンフォールの保存には抗酸化剤の添加が必要であり、エトキシン無添加時の40週後の回収率はそれぞれ 3 及び 15% であった。エトキシン 0.01% 添加で 30 及び 50% に改善され、添加層及び節に応じて徐々に効果もかつ、0.2% 添加により 50 及び 80% に達した。

低温（5 及び -18℃）貯蔵の場合は、5℃におけるβ-カロチノイドが比較的不安定であったが、エトキシン無添加でもよく保存された。5℃におけるβ-カロチノイドの回収率は、無添加時の 40% から 2.0% 添加時の 90% の間に、添加層及び節に応じて等分布に分散した。5 及び 28℃ではエトキシンが同程度分解されたが、-18℃では分解が認められなかった。

キーワード：アルファルファ等の緑葉抽出物。エトキシン、キサンフォール、貯蔵温度、β-カロチノイド。