



Determination of α -, $\gamma(+\beta)$ -, and δ -tocopherols in a Variety of Liver Tissues by Reverse-Phase High Pressure Liquid Chromatography

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ABSTRACT. A modified version of a previously published extraction technique using acetone as the extracting medium and separation on an HPLC equipped with a Spherisorb C₁₈ ODS2 column packed with 3 μ m particles along with a suitable flow rate and mobile phase significantly improves the resolution of the chromatographic output (fluorescence detector), allowing for accurate measurement of α -, δ - and $\gamma(+\beta)$ -tocopherols. It was found that two extractions of the same sample yielded approximately 97% of the total extractable α -tocopherol. Vitamin C was not needed as an anti-oxidant to protect α -tocopherol during extraction from chicken liver but was needed when using fish liver. α -tocopherol was found not to be evenly distributed in beef, chicken or fish liver. COMP BIOCHEM PHYSIOL 113B;1:143–148, 1996.

KEY WORDS. α -tocopherol, δ -tocopherol, $\gamma(+\beta)$ -tocopherol, reverse-phase HPLC, vitamin C, vitamin E, liver

INTRODUCTION

The role of α -tocopherol as an anti-oxidant in the prevention of lipid peroxidation has been studied by several groups (e.g. Burton and Ingold 1986, Di Mascio *et al.*, 1991, Kaiser *et al.*, 1990, Koskas *et al.*, 1984). Some studies have shown that an oxidative attack on liver cells will result in a decrease in their α -tocopherol levels (e.g. Pascoe *et al.*, 1987) while supplementation of cellular α -tocopherol protects the cells against lipid peroxidation (e.g. Pascoe *et al.*, 1987, Pascoe and Reed 1989) suggesting that α -tocopherol is able to reduce the toxic effects, expressed as lipid peroxidation, of certain xenobiotics.

Various extraction procedures have been used to extract α -tocopherol from tissue samples such as dioxane-isooctane (Hung *et al.*, 1980), heptane (Murphy and Kehrer 1987), and methanol-chloroform (Burton *et al.*, 1985). To the authors' knowledge there has been no agreement on the best extraction procedure though it has been shown that acetone is superior to hexane and chloroform/methanol as an extracting agent (Zaspel and Csallany 1983). The problem with acetone extractions has been the large solvent front peak lasting until just prior to the appearance of the α -tocopherol peak when using either a fluorescence detector (Zaspel and Csallany 1983) or a UV detector (Patel *et al.*, 1989), thus hindering the detection of the δ - and $\gamma(+\beta)$ -tocopherol peaks as well

as rendering the accurate measurement of the α -tocopherol peak difficult. Note that the use of an electrochemical detector minimizes the difficulty in detecting δ - and $\gamma(+\beta)$ -tocopherol peaks as well as the α -tocopherol quinone peak (an oxidation product of α -tocopherol) (Pascoe *et al.*, 1987) which appears before δ -tocopherol in reverse phase chromatography. In addition, an electrochemical detector is far more sensitive than either a UV or fluorescence detector.

While the extraction procedure is a determining factor, the particle size in the column used as well as the composition of the mobile phase and flow rate are also important. We present here the advantages of a simple acetone extraction followed by HPLC using a Spherisorb C₁₈ ODS2 column packed with 3 μ m particles and a methanol-water mobile phase and show that such a combination along with a suitable flow rate allows for detection and easy measurement of α -, δ and $\gamma(+\beta)$ -tocopherol peaks. (Note that with the method described here γ and β peaks cannot be separated). For our purposes the simultaneous measurement of all tocopherol isomers is not as important but rather the ability to better quantify the α isomer is, since it is biologically the most active.

The purpose of this study was also to determine if an antioxidant such as vitamin C is needed to protect α -tocopherol during the extraction procedure. It was also of interest to determine whether α -tocopherol is evenly distributed in liver tissues from different species. The answer to this question is important if α -tocopherol determinations are done on only one section of the organ which may not be representative of the whole.

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MATERIALS AND METHODS

Samples

Liver samples from white sucker (*Catostomus commersoni*) were frozen in liquid nitrogen at -196°C and maintained at -145°C until a few days before analysis at which time they were transferred and stored at -25°C .

Liver samples from rainbow trout (*Oncorhynchus mykiss*) grown in a hatchery were dissected from freshly sacrificed specimens, rinsed, and stored in a freezer at -15°C until used, usually 1–2 days.

Dab (*Limanda limanda*) liver samples were taken from fish caught in the North Sea and shipped on dry ice to our laboratory by our colleagues at the Institute for Biological Research, Helgoland, Germany.

Samples of beef and chicken liver were bought at a local store, kept at 4°C and analyzed within a few days.

HPLC System

The HPLC used was a Waters system with a model 600E multisolvent delivery system, a model 715 Ultra Wisp sample processor, a model 484 tunable absorbance detector set at 290 nm and 0.001 absorbance units full scale (AUFS), and a model 470 scanning fluorescence detector set at 290 nm (excitation) and 330 nm (emission). Note that the fluorescence detector was used for all quantification whereas the UV detector was used only for confirmation purposes. The system was controlled by a Maxima 825 software package. The column used was a Chromatographic Sciences Company (CSC) Spherisorb C-18 ODS2 column (10×0.46 cm) packed with $3 \mu\text{m}$ particles. The mobile phase employed was 95% HPLC grade methanol (Burdick and Jackson) to 5% deionized water with the flow rate set at 1.5 ml/min and column temperature set at 22°C . The latter was important given the large temperature fluctuations in our laboratory. Under these conditions the pressure in the column was between 2150–2250 p.s.i. and retention time of α -tocopherol was 7.5 ± 0.2 min based on $N = 13$ days of use. Note that no pre-column was used.

Sample Preparation

Depending upon the goals of the various experiments the procedure was slightly modified. All samples were maintained on ice and in subdued light. In all cases tissue/solvent ratio differences between tubes were avoided. Despite the sensitivity of tocopherols toward light and heat, internal standards were not used for this study. In view of the reproducibility of the method (discussed later), the lack of an internal standard is not critical. The tocopherol concentrations thus presented here are precise but not necessarily accurate. The general procedure was as follows:

A weighed amount of liver (approx. 0.2–0.8 g) was homogenized in the presence of 3–5 times as much acetone (g liver / ml acetone) and 0.5–1.0 ml of 50 mM vitamin C, prepared in methanol. (For any one particular experiment the tissue/

solvent ratio was held constant, but it may vary slightly between experiments). The homogenate was then divided into plastic centrifuge tubes, vortexed for 30 sec, and centrifuged for 10 min at 1500 rpm. An aliquot of the supernatant was filtered through a $0.22 \mu\text{m}$ syringe filter (Millex GV₁₃ by Millipore) into a glass vial and 25 μl were injected into the HPLC. All supernatants were analysed separately.

In cases of multiple extraction the remaining supernatant from above was decanted and 0.5 ml of either methanol or 50 mM vitamin C was added to the pellet followed by 1.0–2.0 ml of acetone. The tubes were then vortexed and centrifuged as above. An aliquot of the supernatant was filtered through a $0.22 \mu\text{m}$ syringe filter into a glass vial and 25 μl were injected into the HPLC. Thus, the supernatants from each extract were analysed separately.

In experiments designed to evaluate the role of vitamin C in protecting α -tocopherol from oxidative degradation, homogenization was carried out in the absence of vitamin C and the homogenate was added to tubes containing 0.5–1.0 ml methanol or 0.5–1.0 ml of a 50–150 mM vitamin C solution.

For experiments addressing the question of whether α -tocopherol is homogeneously distributed in liver, separate homogenates from different parts of one liver sample were made and treated as above.

In attempting to determine the % recovery of exogenously added α -tocopherol, a liver sample was cut up into pieces which were mixed (to avoid heterogeneity problems) and divided into separate centrifuge tubes containing either 1.0 ml of a vitamin C/methanol solution or 1.0 ml of a vitamin C/ α -tocopherol/methanol solution, i.e. a spike. An appropriate amount of acetone was added (based on the weight of liver as indicated above), the sample homogenized, and treated as above.

In all cases, the filtered extracts were kept in sealed glass vials and maintained at 9°C in the autosampler. The samples were run on the HPLC in a random order so as to avoid a time bias.

Standards

α -, δ - and γ -tocopherol standards were prepared by diluting samples obtained from Sigma (St. Louis, MO, USA). Specifically, the products obtained were (\pm) α -tocopherol (approx. 95%), (+) δ -tocopherol (approx. 90%), and (+) γ -tocopherol (approx. 96%). The samples were diluted to the appropriate concentrations in a solution of freshly prepared 50 mM vitamin C dissolved in methanol.

RESULTS

Determination of Optimum Extraction Conditions

During more than two months of work using a variety of livers (chicken, beef, fish), acetone extractions as described above were found to be reproducible, with a mean coefficient of variation of 4.6% ($N = 37$ samples where each sample was

extracted in triplicate to quintuplicate). The detection limit for α -tocopherol is $0.5 \mu\text{g/g}$ liver though in certain cases it can be as low as $0.05 \mu\text{g/g}$ liver. The resolution of the α , $\gamma(+\beta)$, and δ peaks of tocopherol is excellent with no interfering peaks (Fig. 1) thus allowing for accurate quantification of the various isomers of tocopherol.

Number of Extractions Required and % Recovery

Experiments using Dab liver indicate that two extractions of a sample are required to extract $97.7 \pm 2.4\%$ ($N = 5$) of the α -tocopherol present (Fig. 2). A third extraction of the sample yields only $2.4 \pm 0.2\%$ ($N = 4$) of the total α -tocopherol. Thus, a third extraction is not required. Further experimentation using beef, chicken, and White sucker fish livers also suggests that two extractions are sufficient, the second extraction yielding a mean of only $12.8 \pm 7.5\%$ ($N = 22$) of the total α -tocopherol extracted (Fig. 2).

There was a 78–98% (mean = 87%; $N = 5$) recovery of exogenously added α -tocopherol to chicken liver samples in one experiment and 87–91% (mean = 90%; $N = 4$) in another experiment (Fig. 3) so that the overall mean % recovery was $88 \pm 6\%$ ($N = 9$). The α -tocopherol was added prior to homogenization of the liver sample and the recovery data indicate the efficiency of the extraction procedure. The data indicate that as the spike is increased, the % recovery

decreases (Fig. 3), indicating a possible saturation effect of the extraction medium, i.e., acetone. It is possible that this problem may be easily resolved by the addition of more acetone.

Necessity of Vitamin C

Our data suggests that there is no need for vitamin C as an anti-oxidant to protect α -tocopherol in the extraction process when using chicken liver (Table 1). Using the statistical test for the difference between two means (Mendenhall 1987, p. 414), the differences were found to be insignificant even when 150 mM vitamin C was used. When using Dab fish liver it was found that vitamin C is required to prevent the degradation of α -tocopherol.

Homogeneity of Liver Tissues

Extractions using beef, chicken, and fish livers have shown that α -tocopherol is not distributed evenly in liver (Table 2). (Note that only Rainbow trout liver was extracted in 50 mM vitamin C). The values presented for the fish livers are all based on $N = 1$, however, the differences are larger than the long term coefficient of variation for the acetone extractions (4.6%; $N = 37$ samples).

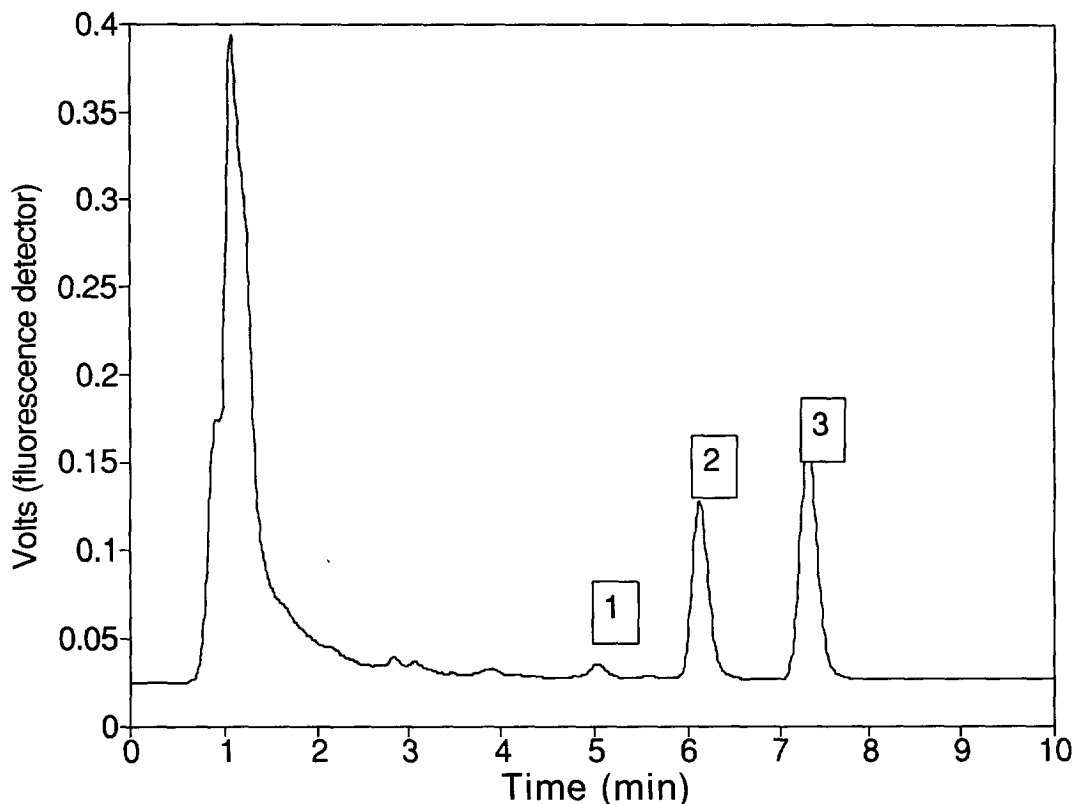


FIG. 1. Chromatographic output (fluorescence detector) for the acetone extraction of a chicken liver. Peaks 1, 2 and 3 are δ -, $\gamma(+\beta)$ -, and α -tocopherol, respectively.

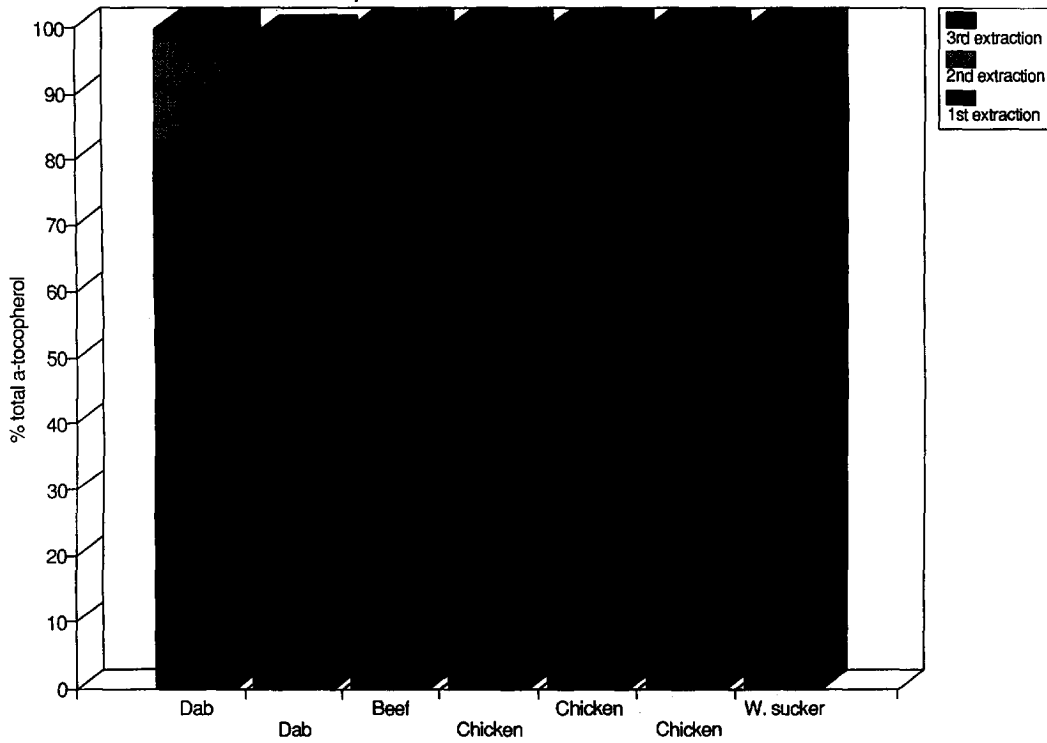


FIG. 2. Percent of total α -tocopherol found during the first, second and third extractions for liver tissues from a variety of species. For Dab fish liver two extractions are sufficient to yield $97.7 \pm 2.4\%$ of the total extractable α -tocopherol.

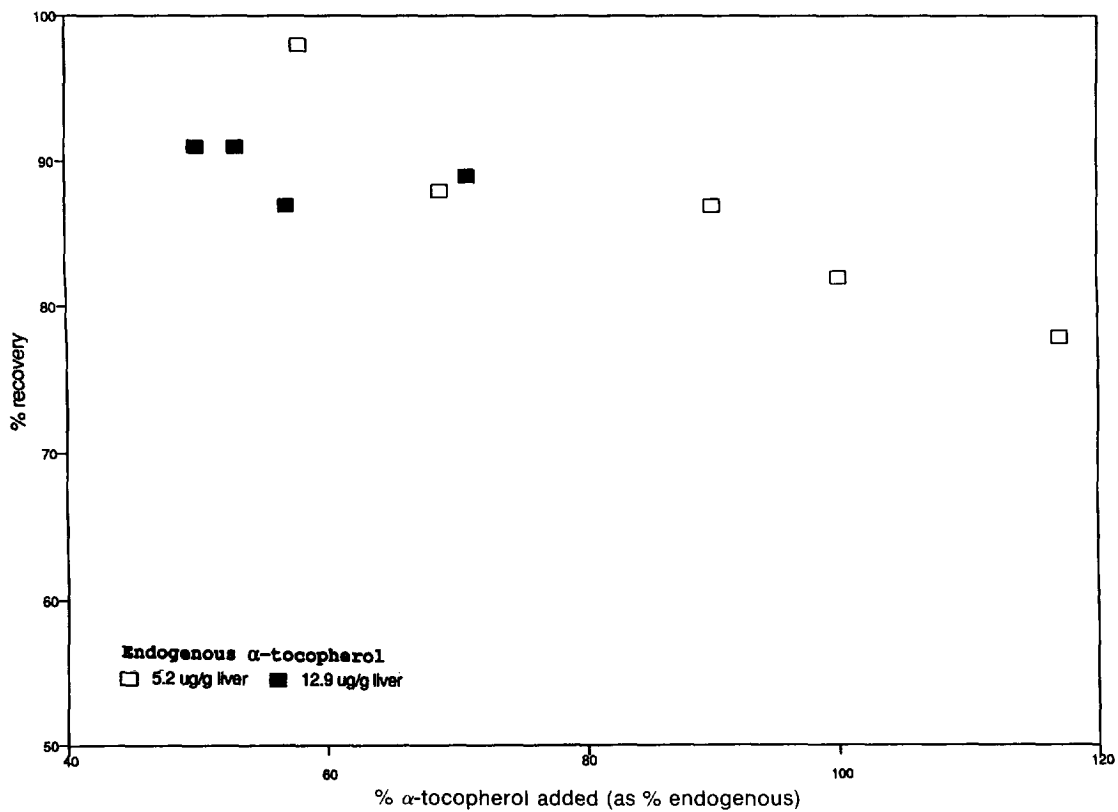


FIG. 3. Percent recovery of α -tocopherol added to samples prior to homogenization. % tocopherol added is expressed as a % of the endogenous α -tocopherol. Mean % recovery is $88 \pm 6\%$ ($N = 9$).

TABLE 1. Comparison of extractions in the presence and absence of vitamin C

| Liver | (. . . . $\mu\text{g } \alpha\text{-T/g liver } \dots\dots)$ | | N | t-calc. | Concl. |
|---------|--|----------------|---|----------------------|--------|
| | Present | VIT C - Absent | | | |
| Chicken | 6.6 \pm 0.1* | 6.5 \pm 0.2 | 5 | 1.858§ | = |
| Chicken | 6.7 \pm 0.2† | 6.5 \pm 0.2 | 5 | 1.5177 | = |
| Chicken | 6.8 \pm 0.3‡ | 6.5 \pm 0.2 | 5 | 1.594 | = |
| Dab | 71.5 \pm 0.7‡ | 64.3 \pm 2.3 | 3 | 5.567 [¶] | ≠ |

All values are reported as a mean \pm standard deviation.

T-calc. is t-calculated for the statistical test for the difference between two means.

*: 50 mM vitamin C

†: 150 mM vitamin C

‡: 100 mM vitamin C

§: t-critical = 1.895 for $\alpha = 0.05$ and 7 degrees of freedom.

^{||}: t-critical = 1.860 for $\alpha = 0.05$ and 8 degrees of freedom.

[¶]: t-critical = 2.353 for $\alpha = 0.05$ and 3 degrees of freedom.

DISCUSSION

Determination of Optimum Extraction Conditions

One study (Patel *et al.*, 1989) using rat liver samples found the need for a purification step with a Sep-Pak C₁₈ cartridge prior to HPLC separation using a C₈ column (5 μm particle size) to avoid a large solvent front containing interfering substances which makes α -tocopherol detection difficult. Despite this treatment the solvent front peak still hinders the determination of δ - and $\gamma(+\beta)$ -tocopherol peaks. In our method a 0.22 μm filter is used prior to HPLC to eliminate any particles which may clog the column. Otherwise the two extraction methods are the same however, the chromatographic conditions are different (Table 3).

A previous study (Zaspel and Csallany 1983) also used acetone as the extraction medium, however, the extracts of multiple washings were combined, reduced in volume by drying under N₂ at 30–40°C and then filtered through a 0.45 μm filter. A C₁₈ column (10 μm particle size) preceded by a RP-18 guard column and a mobile phase of 2% water in methanol at a flow rate of 2 ml/min was used (Table 3). A large solvent

front was apparent, its tail ending just prior to the beginning of the α -tocopherol peak thus hindering the determination of δ - and $\gamma(+\beta)$ -tocopherol peaks.

Previous work has shown that the smaller the particle size employed in the column packing the better the resolution and efficiency of that column (Ball 1988). The particle size used in the present study was 3 μm compared to the 10 and 5 μm particle sizes used by the other studies, respectively (Zaspel and Csallany 1983, Patel *et al.*, 1989). Our experience has shown that changes in the composition of the mobile phase, for example, using 100% methanol instead of 95% methanol:5% water, will result in peak crowding and shorter retention times, however, the solvent front will still not interfere with the tocopherol peaks. Increasing the flow rate from 1.5 ml/min to 2.0 ml/min will result in shorter retention times, however, the relative retention times remain constant so that all the tocopherol peaks are easily identified. Thus particle size appears to be a critical factor.

Since acetone extractions of tissues were found to be superior to other solvent systems (Zaspel and Csallany 1983), our findings should eliminate any hesitation to the use of acetone extractions due to solvent front interferences in the chromatographic separation. The advantage of an acetone extraction lies in its relative simplicity, with fewer manipulative steps required. As such, it provides an attractive alternative to other more cumbersome methods where drying and preparative clean-up are required.

Necessity of Vitamin C

The reason why the extraction of α -tocopherol from chicken livers does not require vitamin C to prevent degradation of the α -tocopherol whereas Dab fish livers do may be related to the possible presence of residual vitamin C in chicken livers. Chickens are able to synthesize vitamin C whereas many fishes, including Dab, cannot and thus the presence of vitamin C in fish liver is related to diet. The fact that commercially

TABLE 2. Results (mean \pm SD) show that α -tocopherol is not evenly distributed in beef, chicken and fish livers. Only trout liver extractions were done in the presence of 50 mM vitamin C

| Liver | Extract | $\mu\text{g } \alpha\text{-T/g liver}$ | N | t-calc. | Concl. |
|---------------|----------|--|---|-----------------|--------|
| Beef | A-end | 8.9 \pm 1.5 | 6 | A vs B: -2.869* | ≠ |
| | B-end | 12.4 \pm 2.6 | 6 | | |
| Chicken | A-end | 22.7 \pm 0.9 | 5 | A vs B: 3.363† | ≠ |
| | B-middle | 25.2 \pm 1.4 | 5 | B vs C: 4.050† | ≠ |
| | C-end | 22.5 \pm 0.5 | 5 | A vs C: 0.383† | = |
| Rainbow trout | A-end | 134 | 1 | | |
| | B-middle | 111 | 1 | | |
| | C-end | 141 | 1 | | |
| White sucker | A | 143 | 1 | | |
| | B | 190 | 1 | | |

Where available, the part of the liver extracted is indicated.

N is the number of extractions used.

T-calc. is t-calculated for the statistical test for the difference between two means.

*t-critical = -1.812 for $\alpha = 0.05$ and 10 degrees of freedom

†t-critical = 1.860 for $\alpha = 0.05$ and 8 degrees of freedom

TABLE 3. Comparison of the chromatographic conditions for three studies, including this one, using acetone as the extraction medium

| | Present study | Zaspel & Csallany (1983) | Patel et al. (1989) |
|--------------------|--------------------------|--------------------------|---------------------|
| Packing | Spherisorb C-18, ODS2 | LiChrosorb RP-18 | Ultrasphere C-8 |
| Column L × W (cm) | 10 × 0.46 | 25 × 0.46 | 25 × 0.46 |
| Particle size | 3 μm | 10 μm | 5 μm |
| Mobile phase | 95% methanol 5% water | 98% methanol 2% water | 100% methanol |
| Flow rate (mL/min) | 1.5 | 2.0 | 1.5 |

produced poultry are fed a diet containing vitamin E supplements whereas fish from a natural environment are not may also have an effect. If the method is to be standardized then an antioxidant such as vitamin C should be used.

Homogeneity of Liver Tissues

The finding that α -tocopherol is not evenly distributed in fish (two species), beef, and chicken livers underlines the importance that experiments dealing with α -tocopherol in liver should use a representative liver sample to avoid obtaining potentially inaccurate results.

CONCLUSION

This study has found that extracting tocopherol from a variety of livers using acetone as the extracting medium and an HPLC equipped with a Spherisorb C₁₈ ODS 2 column packed with 3 μm particles allows for easy quantification of α -, δ - and γ (+ β)-tocopherol. With this method two extractions of a sample are sufficient to yield about 97% of the extractable tocopherol in fish liver and a similar percentage in beef and chicken liver.

Vitamin C was needed to prevent the oxidation of α -tocopherol during extraction of fish liver but not chicken liver probably due to a combination of differences in the diets of the two species (chickens are fed a vitamin E supplemented diet) and a potentially higher residual vitamin C concentration in chicken livers as chickens are able to synthesize their own vitamin C whereas most fish cannot.

It was found that α -tocopherol is not evenly distributed in fish, chicken, or beef liver. The importance of this last finding suggests that a representative sample of liver must be used to obtain accurate measurements of α -tocopherol for the whole of the liver tissue.

Finally, this method may allow for the quantification of all 3 tocopherol isomers in other tissues such as fish muscle where other studies have found α and γ isomers (e.g. Erickson 1992). The importance of quantifying not only the α isomers but also the δ and γ isomers may be important for a better understanding of conversions between δ , γ and α isomers and have implications for ecophysiological and aquaculture research.

The authors wish to thank Jacques Bureau and Anne-Marie Prud'homme of the Centre Saint-Laurent for supplying the White sucker liver samples and the crew of the research vessel FK Uthörn at the Institute for Biological Research, Helgoland, Germany for providing help in the sampling of the North Sea Dab. Funding support was provided through the Canada-Germany Science and Technology agreement.

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