

Selective Extraction of Phospholipid Mixtures by Supercritical Carbon Dioxide and Cosolvents

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ABSTRACT

To optimize and enhance the value of a previously developed supercritical fluids (SF) process for removing oil from soybean flakes, we devised a two-step, sequential scheme for extraction of oil and phospholipid-containing fractions using SC-CO₂ alone or with ethanol. PLs were selectively removed from the flakes using the SC-CO₂/ethanol mixture. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were more readily solubilized in the SC-CO₂/cosolvent mixtures than were phosphatidylinositol and phosphatidic acid. The extent of recovery of PC and PE was a function of the molar fraction of cosolvent. Some fractionation of constituent phospholipids could be achieved by varying the molar fraction of ethanol. The extracts from the SF process were characterized by inductively coupled plasma spectroscopy (ICP) and HPLC with evaporative light scattering detection.

Key Words: extraction, supercritical fluid, cosolvent, phospholipids, soybean oil

INTRODUCTION

PHOSPHOLIPID (PL) MIXTURES are traditionally obtained from a by-product associated with the seed oil processing industry, i.e. lecithin from the soybean oil refining process. Supercritical carbon dioxide (SC-CO₂) extraction of seed oils leaves much of the PL content of the seeds in the deoiled meal, whereas in the traditional refining process, the PLs are partially partitioned into the hexane-extracted oil. Phospholipids (PLs) are polar conjugate lipids (Horrocks, 1989; Cherry and Kramer, 1989) and commonly occur in plants (Scholfield, 1985). Cherry and Kramer (1989) noted that several researchers often use the terms lecithin and PC interchangeably. However, lecithin refers commercially to a complex natural mixture of PLs, traditionally obtained by water-washing crude vegetable oil and separating and drying the hydrated gums (Scholfield, 1985). Therefore, "lecithin" is often used to describe a diverse group of commercially available PL mixtures including fractions containing one or more PLs, triglycerides, pigments, carbohydrates, sterols, cerebrosides, in different proportions (Scholfield, 1985; Flider, 1985).

Several different kinds of lecithin are commercially available. Some principal uses are: health and human nutrition (Zeisel, 1989), food processing (Dashiell, 1989), baking (Knightly, 1989), formulating beverages (Sander, 1989), confectionery products (Appl, 1989), enhancing animal health and nutrition (Kullenberg, 1989), and some nonfood applications (Schmidt and Orthofer, 1985; Baker, 1989; Sipos, 1989; Chagnon and Ferris, 1989). In the U.S. production of PL mixtures (lecithin) has continually increased (Douvelis, 1994). These markets will continue to increase with development of highly purified PL extracts in the cosmetic, microencapsulation, and liposome fields.

List et al. (1989) showed that supercritical carbon dioxide (SC-CO₂) was very effective in removing oils from seed matri-

ces, devoid of appreciable PL content. This was exploited by List et al. (1993) and Eggers and Wagner (1993) to continuously degum pre-extracted soybean oil and lecithin, respectively. However, the limited solubility of PLs in SC-CO₂ leaves a potentially valuable by-product in the spent seed matrix that if recovered, could be of economic advantage.

The SFE process is a particularly appropriate process, since the extracting agent is environmentally acceptable and non-toxic in foods. Since neat CO₂ will not effectively solubilize PL moieties, the choice of a suitable cosolvent must be based on thermodynamic considerations and with regard to its food safety, that is, it should be "Generally Recognized As Safe" (GRAS).

A logical choice for a cosolvent would be ethanol, Schneider (1989) and Gober et al. (1993) have reviewed the alcohol-based fractionation of PLs. Prosis (1985) noted that ethanol was an excellent solvent for isolating PLs for food use. Neat ethanol fractionation of a SC-CO₂ deoiled lecithin concentrate was optimized by Manohar et al. (1995). Temelli (1992) used SC-CO₂/ethanol mixtures to qualitatively demonstrate the removal of phospholipids from canola seed. Dunford and Temelli (1995) expanded on those initial studies using ethanol as a "cosolvent" after initial deoiling of canola meal. Only 21% of PLs were recovered at 55 MPa and 70°C using 8 mole % of ethanol. Ethanol presoaking of the above meal as well as an acetone-insoluble isolate enhanced recovery of the PLs, but no more than 50% yield was realized. Since high pressure phase equilibria data are available for ethanol/CO₂ mixtures (Kobe and Lynn, 1953; Gurdial et al., 1991), we focused our efforts on use of ethanol cosolvent for extracting PL mixtures at higher pressures and cosolvent addition levels than reported by (Dunford and Temelli, 1995).

Our objectives were: (a) to develop an integrated procedure for removal of oil and phospholipid fractions from soybean flakes using SC-CO₂ and ethanol with potential for scaleup and (b) to solubilize the lecithin fraction into the SC-CO₂/cosolvent mixture to further study the fractionation of PLs.

MATERIALS & METHODS

Materials

Soybeans were provided by Illinois Crop Improvement Association, Inc. (Williams 82 Soybeans, Lot 4849, Producer # 14290). Analyses showed a basic composition of 21.20 wt % of oil 8.30 wt % moisture, and 5.072 mg/g total phosphorus. The analyses were performed using the following chemical reagents: ethanol - 100% dehydrated, U.S.P. punctilious® (Quantum Chemical Corporation, USI Division, Tuscola, IL); petroleum ether (Baker Analyzed® Reagent, Phillipsburg, NJ); benzene (Omni Solv® - EM Industries, Inc., Gibbstown, N.J.); HPLC-grade chloroform (Fisher Scientific, Fairlawn, NJ), and nitric acid from Fisher Scientific, Fairlawn, NJ). The CO₂ utilized in the extractions was a welding grade from National Welding Supply (Bloomington, IL).

Methods

SC-CO₂ extractions. The extractions were carried out using a two-stage extraction sequence:

Soybean Flakes (SBF)

↓

SC-CO₂ EXTRACTION → Soybean Oil (OIL)

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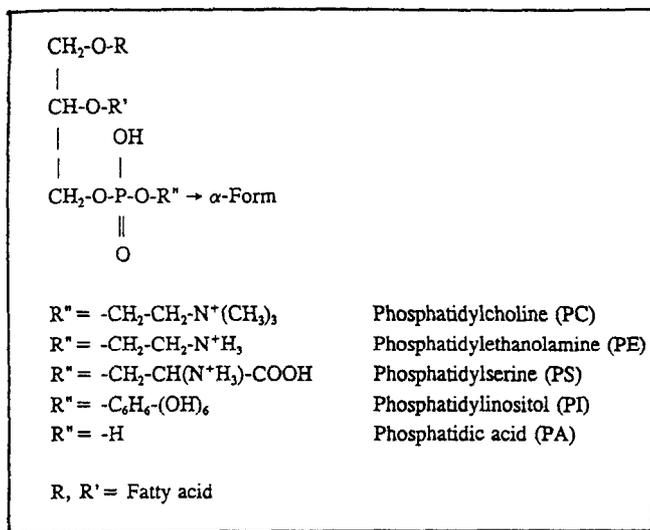


Fig. 1—Commonly occurring phospholipids in plants.

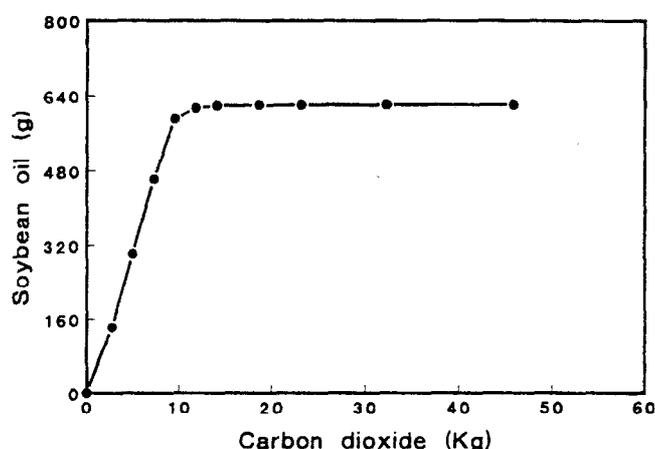


Fig. 2—Extraction profile for oil removal from 3 kg lot of soybean flakes

(70 MPa, 80°C)

↓

Defatted Soybean Flakes (DSF)

↓

SC-CO₂/ETHANOL EXTRACTION → Phospholipid-Enriched Fraction (PLF)

(62.8 MPa, 80°C)

X_{Eth}: 0.05, 0.1, 0.15, 0.2

↓

Exhausted Oilcake (EO)

The first extraction was performed using a SFE pilot plant (Friedrich et al., 1988) using 3 kg of soybean flakes placed in a 4L vessel. The extraction of soybean oil with SC-CO₂ was performed at 70 MPa and 80°C; optimal conditions for rapid extraction of seed oils (List et al., 1989). The extracted oil was phase separated at the back pressure relief valve (BPRV) by reducing the pressure to 17.2 MPa to allow precipitation of oil in the receiver vessel (R). The decompressed CO₂ was then recirculated to the compressor.

After the passage of ≈13 kg of CO₂, the maximum amount of oil is removed from the flakes (Fig. 2). Upon completion of extraction, 622g of DSF were recovered. The DSF were then stored at -29°C until a second extraction was performed. This lot of DSF served as a uniform source feedstock for the second sequence of extractions (CO₂ with ethanol).

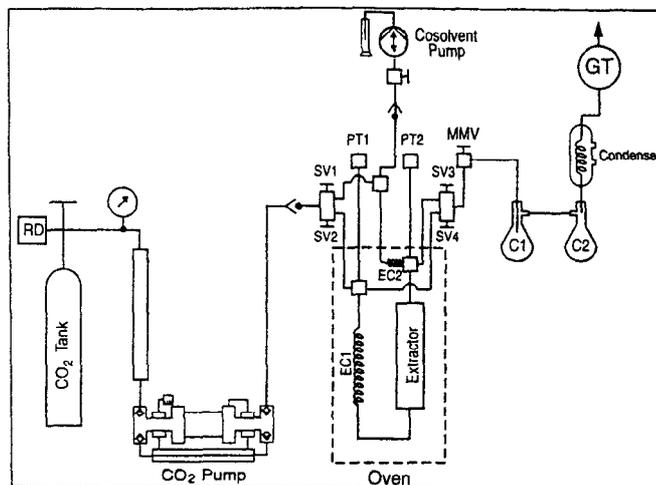


Fig. 3—Supercritical fluid extraction screening system with cosolvent addition option.

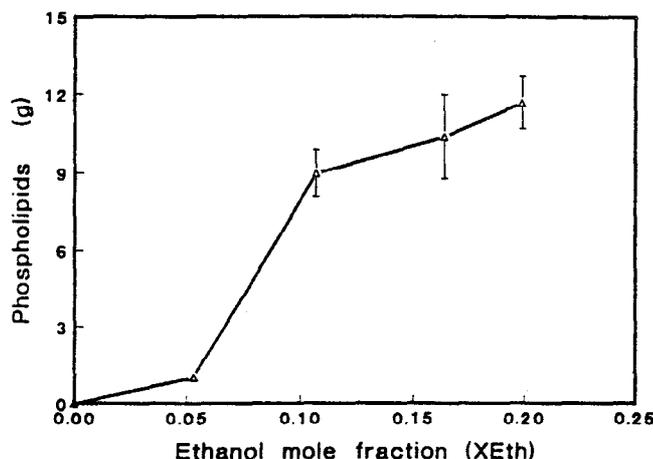


Fig. 4—Phospholipid yield per kg of soybean flakes as related to ethanol concentration in the supercritical fluid phase. RD = rupture disk, SV = switching valve, PT = pressure transducer, EC = equilibration coil, MMV = micrometering valve, C1, C2 = collection vessels, GT = gas totalizer.

Extractions were also performed with a modification of an apparatus (Fig. 3) previously used in our laboratory (Favati et al., 1991), but changed to permit cosolvent. The CO₂ was delivered by a Haskel gas booster pump (Model AGT 62-152, Haskel, Inc., Burbank, CA) through a check valve to a two-way switching valve (SV1/SV2). From SV1 it entered a three-way valve where ethanol could be added for the second stage extraction via a HPLC pump (Model 100A, Beckman Instrument, Inc., Fullerton, CA). The CO₂ or CO₂/cosolvent mixture then passed through an equilibration coil (EC2) inside a temperature controlled GC oven. Both extraction fluids passed downward through the soybean flakes contained in the extraction vessel, exiting at the bottom of the extractor and flowing through another switching valve (SV3/SV4). The dissolved extract passed through a heated micrometering valve (Series 30VRMM, Autoclave Engineers, Erie, PA) into the first of two collection flasks (C1). Ethanol entrained in the decompressed CO₂ stream was further separated with the aid of a second flask (C2) and a jacketed, cooled condenser. Total and intermediate volumes of CO₂ were ascertained using a gas totalizer (Model DTM200A, American Meter Division, Philadelphia, PA).

Experiments were conducted on samples of ≈ 60g; the seeds were ground and thinly flaked (0.1–0.25 mm) to enhance the yield of oil, in accordance with findings of Snyder et al. (1984). The second sequence of extractions was carried out at 68.2 MPa, 80°C, using 80 mL of ethanol (63.2g) for each run. The ethanol molar fraction (X_{Eth} = 0.05–0.20) was changed by altering its flow rate (0.44–2.67 mL/min) and/or the CO₂

Table 1—Mass balance flow sheet for supercritical fluid extractions at different ethanol mole fractions

$X_{Eth} = 0.053 \pm 0.002$										
	IN					OUT				
	W.M.	D.M.	FAT	WATER	PHOSPHORUS	W.M.	D.M.	FAT	WATER	PHOSPHORUS
	g	g	g	g	mg	g	g	g	g	mg
SBF	1000	916	212	83	5072	207	203	203	4	(≤ 0.2)
OIL										
PLF						3 \pm .7	3 \pm .7	1 \pm .08	—	32 \pm 5
EO						707 \pm 10	686 \pm 5	1 \pm .4	21 \pm 5	5084 \pm 34
Total	1000	916	212	83	5072	917 \pm 10	892 \pm 5	205 \pm .5	25 \pm 5	5116 \pm 29
Δ in g						-83 \pm 10	-24 \pm 5	-7 \pm .5	-58 \pm 5	+44 \pm 29
Δ in %						-8 \pm 1	-3 \pm .5	-3 \pm .2	-62 \pm 9	+9 \pm .6
$X_{Eth} = 0.107 \pm 0.008$										
	IN					OUT				
	W.M.	D.M.	FAT	WATER	PHOSPHORUS	W.M.	D.M.	FAT	WATER	PHOSPHORUS
	g	g	g	g	mg	g	g	g	g	mg
SBF	1000	916	212	83	5072	207	203	203	4	(≤ 0.2)
OIL										
PLF						10 \pm 1.6	10 \pm 1.6	8 \pm .7	—	301 \pm 31
EO						701 \pm 4	680 \pm 5	1 \pm .1	23 \pm 1	4786 \pm 19
Total	1000	916	212	83	5072	918 \pm 4	893 \pm 5	212 \pm .7	27 \pm .9	5088 \pm 50
Δ in g						-82 \pm 4	-23 \pm 5	+5 \pm .7	-56 \pm .9	+16 \pm 50
Δ in %						-8 \pm 1	-3 \pm .5	+2 \pm .3	-62 \pm .8	+3 \pm 1
$X_{Eth} = 0.164 \pm 0.004$										
	IN					OUT				
	W.M.	D.M.	FAT	WATER	PHOSPHORUS	W.M.	D.M.	FAT	WATER	PHOSPHORUS
	g	g	g	g	mg	g	g	g	g	mg
SBF	1000	916	212	83	5072	207	203	203	4	(≤ 0.2)
OIL										
PLF						14 \pm 3	14 \pm 3	8 \pm 1	—	348 \pm 53
EO						709 \pm 12	636 \pm 13	1 \pm .1	26 \pm .7	4726 \pm 60
Total	1000	916	212	83	5072	931 \pm 10	853 \pm 11	212 \pm 1	30 \pm .7	5074 \pm 72
Δ in g						-69 \pm 10	-15 \pm 11	+2 \pm 1	-53 \pm .7	+2 \pm 72
Δ in %						-7 \pm 1	-2 \pm 1	+1 \pm .6	-64 \pm .9	<.1 \pm 1.4
$X_{Eth} = 0.199 \pm 0.007$										
	IN					OUT				
	W.M.	D.M.	FAT	WATER	PHOSPHORUS	W.M.	D.M.	FAT	WATER	PHOSPHORUS
	g	g	g	g	mg	g	g	g	g	mg
SBF	1000	916	212	83	5072	207	203	203	4	(≤ 0.2)
OIL										
PLF						18 \pm 3	18 \pm 3	10 \pm 1	—	391 \pm 33
EO						701 \pm 5	676 \pm 3	1 \pm .1	25 \pm 8	4699 \pm 73
Total	1000	916	212	83	5072	926 \pm 3	897 \pm 6	214 \pm .9	29 \pm 8	5090 \pm 40
Δ in g						-74 \pm 3	-19 \pm 6	+2 \pm .9	-54 \pm 8	+18 \pm 40
Δ in %						-7 \pm .3	-2 \pm .6	+1 \pm .4	-66 \pm 9	+3 \pm .8

flow rate (1.60–4.00 L/min as measured at ambient conditions). Mole fractions of ethanol at 0.05, 0.10, 0.15, and 0.20 were used for extraction/fractionation of the PL-laden fractions. Three separate extractions were performed at each concentration and the statistical average and standard deviation were determined for extractions under each set of conditions.

Chemical analyses

To develop a mass balance flow-sheet, analyses were performed on the SBF, DSF, OIL, PLF, and EO including total weight, dry matter (AOCS, 1988, Method BC 2-49), crude fat (AOCS, 1988, Method BC 3-49), and total phosphorus (AOCS, 1988, Method BC 5-49), for which samples were ashed and dissolved in 5% HNO₃. Phosphorus was determined by ICP (Model 400, Plasma Emission Spectrometer, Perkin Elmer, Norwalk, CT).

Analytical separations were performed with a Spectra-Physics (Thermo-Separation Products, Fremont, CA) Model 8800 HPLC using a Rheodyne 7125 injector (50 μ L loop), and a Varex Model ELSD II evaporative light scattering detector (ELSD) (Alltech Associates, Inc., Deerfield, IL) Model ELSD II. The analog signal from the ELSD was interfaced to a PC 1000 computer system (Spectra-Physics). The phospholipid fractions were analyzed using a normal phase HPLC column, a Lichrospher Si 60/II, 3 μ m particle size (250 \times 4 mm i.d.) column (EM Separations, Gibbstown, NJ). Separation was achieved using a linear gradient program consisting of chloroform:tert-butyl methyl ether (750:150 v/v) to methanol:ammonium hydroxide:chloroform (920:70:10 v/v) over 30 min, with an isocratic hold of 10 min. The flow rate was

0.5 mL/min (Abidi, 1994; Mounts et al., 1992). PLFs were dried and redissolved in 3 mL chloroform before HPLC.

PLs were extracted from SBF, EO, and DSF by a threefold extraction using the solvent mixture of Bollmann (1:1:1, ethanol: petroleum ether: benzene) (Bollmann, 1923; Wittcoff, 1951), then dried, and redissolved in chloroform. This permitted determination of the total PL content of the original soybean samples or PL not extracted, in the case of the SFE. The PL content (mg PL/g DSF) for individual PLs in the DSF were PE (0.68), PI (0.89), PA (0.45) and PC (2.74).

RESULTS & DISCUSSION

THE MASS BALANCE was compared for four extractions (Table 1) using different mole fractions of ethanol. These were normalized to 1 kg of SBF for the neat SC-CO₂ extraction, as well as different ethanol cosolvent additions used in the second extraction sequence ($X_{Eth} = 0.053, 0.107, 0.164,$ and 0.199 , respectively). There was generally an insignificant difference in mass balances. The high Δ s for the wet matter (W.M.) were due to water loss via entrainment in the CO₂. Hence the mass balances for water would be in error, due to some loss from the receiver vessel. However, the mass balances of the dry matter (D.M.) were acceptable. Further confirmation of this is seen from comparing the Δ s for the D.M. and the difference in Δ s between W.M. and water. Here Δ s for the W.M. - Δ s for cor-

Table 2—% Recovery of phospholipids from deoiled soybean flakes as a function of ethanol concentration in SC-CO₂

mole % Ethanol in SC-CO ₂	% Recovery of phospholipids ^a			
	PE	PI	PA	PC
5	16	16	0	16
10	77	47	64	91
15	89	62	72	94
20	91	69	71	94

^a PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PA = Phosphatidic acid; PC = Phosphatidylcholine.

responding water = Δs for D.M.. The mass balance accounting of oil and phosphorus content indicated that extractions could be well characterized. The highest average % error in the mass balance for the phosphorus, crude fat, and D.M. were 0.9, 3.0, and 3.0 %, respectively.

The total phosphorus in soybeans could be converted to total PL basis by using a conversion factor of 30 (AOCS, 1973). The total amount of PLs recovered from each of the above extractions were determined by varying the amount of ethanol in the second one based on the conversion factor (Fig. 4). At $X_{E_{th}} = 0.052$, the recovery was very low and precise (the error bar was smaller than the symbol code, Fig. 4); however, PL recoveries could be increased considerably using mole fractions of ethanol corresponding to 0.10 or larger. For example, from 1 kg of SBF using SC-CO₂/ethanol mixtures with $X_{E_{th}} = 0.05, 0.10, 0.15,$ and 0.20, it would be possible to extract 1.0, 9.0, 10.4, 11.7g of PLs, respectively.

Data from a large number of HPLC runs were used to determine the PL content of PLF and EO fractions (Table 2). The data were normalized to 100% by considering the total amount of each individual PL distributed between the PLF or EO. The % recovery was defined as wt fraction of a PL in the PLF/wt fraction of a PL in the PLF + wt fraction of a PL in the EO. Increasing the amount of ethanol in the SC-CO₂ increased the amount of each individual PL extracted (Table 2). For $X_{E_{th}}=0.05$, only a very small amount of each individual PL was extracted; however, by increasing the $X_{E_{th}}$ to 0.10, it was possible to extract 91% of the total amount of PC, 77% of PE, 64% of PA, and only 47% of PI. These recoveries could be improved by increasing the mole fraction of ethanol in the SC-CO₂ to 0.15, the PE recovery increasing to 89%, while PA and PI would be recovered at 72 and 62% levels. Increasing the $X_{E_{th}}$ to 0.20 did not result in any increase in three of the major soybean PLs, except PI's recovery was improved to 69%. Overall the data indicate that relative to other studies (Dunford and Temelli, 1995), both high pressure CO₂ (70 MPa) and cosolvent were required to quantitatively recover soybean PLs from the EO. The addition of a preferred cosolvent only, would not be adequate to affect high recovery.

PC and PE are generally extracted in preference to PA and PI; however, the enrichment factors into the supercritical fluid phase from the EO are small at $X_{E_{th}}=0.05$. The addition of $X_{E_{th}}=0.10$ to the SC-CO₂ made recovery of the soya PLs attractive, however the relative separation factors would permit only limited fractionation. The greater apparent solubilization of PC relative to the other PLs may be due to its lower estimated cohesive energy density, 95.45 cal/cc (Fedors, 1974). The relative lower recovery of PI reflects its more polar and acidic nature (cohesive energy density = 145.2 cal/cc), which makes it more difficult to solubilize in the SC-CO₂/ethanol at $X_{E_{th}}=0.10$. The choice of ethanol as cosolvent (having a cohesive energy density 162.8, Barton, 1991), enhanced the solubilization of the more polar PL during the SFE. However, the high levels of recovery attained between $X_{E_{th}} = 0.10-0.20$ suggest that SC-CO₂ with ethanol as a cosolvent might be combined with chromatographic fractionation, i.e., supercritical fluid chromatography (SFC) to provide additional fractionation.

CONCLUSION

A CONSECUTIVE, TWO STEP SFE PROCESS could be used to deoil vegetable seeds as well as isolate phospholipid-enriched frac-

tions. The deoiling could be accomplished using neat SC-CO₂ extraction followed by a SC-CO₂/ethanol extraction. The molecular specificity of the SC-CO₂/ethanol extractions for PLs was similar to results using alcoholic liquid solvent extraction. Our method has the possibility of producing industrially-useful PL mixtures varying in PL content, particular when combined with preparative SFC techniques.

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prepared in a way to prevent an excess of lysine (raw tuna + wheat flour). Both the BV and the NPU decreased (Table 5). Other studies have reported analogous results with evaporated condensed milk (Bender, 1984), because lysine is in relative surplus both in raw and processed milk. The BV was unchanged, but when milk was mixed with certain proportions of cereal, limited by lysine, the mixture indicated protein damage.

CONCLUSION

CANNING caused an increase in protein, ash and fat content in canned tuna compared to raw tuna. In particular fat and ash increase appeared to be higher for longer sterilization times because a longer contact led to a higher absorption of fill oil and added salt. However available lysine content decreased when sterilization time was extended. Protein Digestibility, BV and NPU remained unchanged when the sole protein source in the diet was tuna protein. When the tuna was used as a valuable supplement to protein limited in lysine, the canning process diminished the nutritional quality of tuna protein. Therefore, sterilization time should not be prolonged.

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