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Alessio N. Serreqi, Colette Breuil and John N. Saddler

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Sustainable Forest Management Network
G208 Biological Sciences Building
University of Alberta
Edmonton, Alberta, T6G 2E9
Ph: (780) 492 6659
Fax: (780) 492 8160
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Use of Immunoassays to Monitor In –Mill Concentrations of Dehydroabietic Acid

SFM Network Project: Use of Immunoassays to Monitor In –Mill and Waster Water Concentrations of Hydrophobic Organic Material

by

Alessio N. Serreqi, Colette Breuil and John N. Saddler

Chair of Forest Products Biotechnology, Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z4

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ABSTRACT

The pulp and paper industry is moving towards mill closure for environmental and economic reasons. As mill waters are recycled, extractives concentrations are expected to increase significantly and methods to monitor their levels will be needed. One class of extractives of concern is the resin acids because of their toxic nature and detrimental effects to pulp and paper making processes. A simple, accurate, fast, reliable analytical method will be necessary to monitor resin acid levels throughout pulp mill process waters. An enzyme-linked immunosorbent assay (ELISA) for dehydroabietic acid (DHA), a resin acid from the abietane class, was developed using polyclonal antibodies raised in rabbits. These antibodies were very sensitive for DHA, with an IC_{50} of 7 ± 2 ng/mL (ppb). Characterization of the antibodies revealed cross-reactivity with the abietanes; abietic and palustric acid by 54% and 30%, respectively. The effect on the antibodies of other extractives such as fatty acids, fatty alcohols, mono-, di-, and triglycerides, sterols, steryl esters and waxes, present in process waters was also tested and determined to be minimal. Direct analyses of resin acid-free process water samples spiked with known amounts of DHA, however, revealed that components present in the samples interfered with the ELISA. Therefore, a simple solid phase extraction (SPE) pretreatment on an aminopropyl column was developed to remove the interfering components. Recoveries of DHA spiked process water samples after the SPE pretreatments were determined by ELISA and HPLC to be essentially 100%. However, the high variability of the ELISA results along with the observed cross-reactivities with other resin acids make it better suited as a screening method for high resin acid levels in pulp mill process waters.

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INTRODUCTION

Pulp and paper mills worldwide are moving towards water systems closure for economic and environmental reasons. Dissolved and colloidal substances (DCS) are known to be released from wood during mechanical pulping processes and accumulate in the white water system (Allen 1975). One consequence of closure is elevated level of DCS, therefore, mills will require increased management of their in-mill water systems to maintain productivity and product quality (Farlow 1996). The dissolved and colloidal substances are mostly composed of lipophilic extractives, lignins, polysaccharides and inorganic materials (Sjostrom 1990; Ekman, et al. 1990). High levels of lipophilic extractives are associated with pitch problems, which affect machine runnability and product quality in a pulp and paper mill.

Resin acids are a class of lipid extractives associated with pitch (LeRoux, et al. 1997). They also decrease water surface tension and wet-web strength thus slowing the paper making processes (Zhang, et al. 1999) and are toxic to aquatic life. Their 96h-LC_{50} to rainbow trout ranges from 0.2 to 1.7 mg/mL at neutral pH (McLeay and Associates Ltd. 1986), therefore, monitoring resin acid levels in process lines of mills moving toward water systems closure will become increasingly important. Pulp and paper mills using a softwood furnish release resin acids from wood chips into process waters regardless of the pulping method (McLeay and Associates Ltd. 1986). The eight most common resin acids found in Canadian softwood pulp mill waters are divided into two groups: the abietanes and the pimaranes. Dehydroabietic acid (DHA), abietic acid, palustric acid, levopimaric acid, and neoabietic acid are abietanes while the pimaranes consist of sandaracopimaric acid, isopimaric acid and pimaric acid (figure 1).

Gas chromatography (GC) is the method of choice for monitoring the levels of individual resin acids and several methods using flame ionization detection have been developed for analysis of pulp mill effluents (NCASI 1986; Voss and Rapsomatiotis 1985). GC equipped with a mass spectrometry detector has also been used to measure resin acids in pulp mill effluents (Dethlefs and Stan 1996). Unfortunately these techniques are tedious and require extensive sample pretreatment. The samples must be extracted, purified on a solid phase extraction column, then derivatized before they are finally quantified. Large numbers of samples cannot be routinely and rapidly analyzed and the above methods are not amenable to on-line analysis.

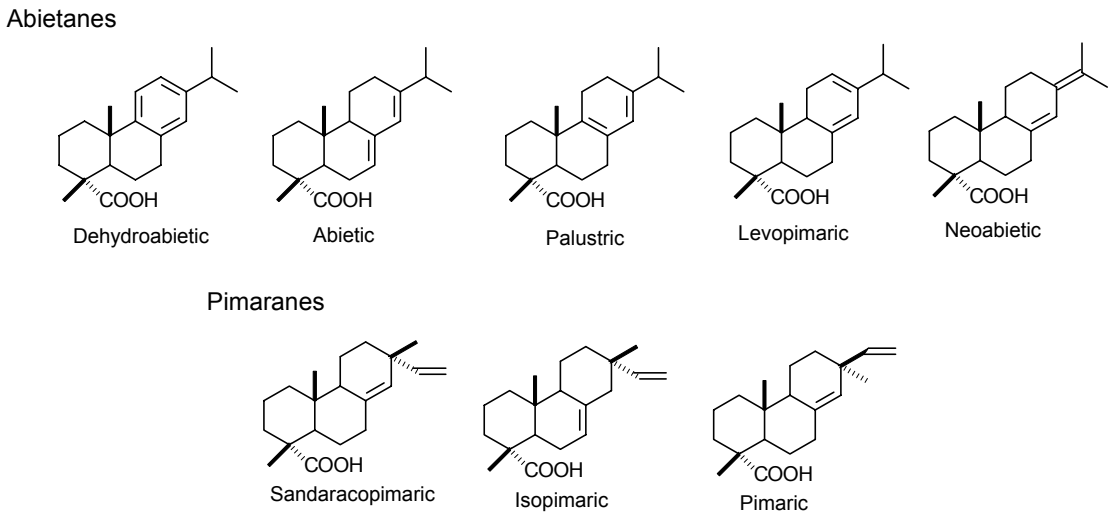


Figure 1. The eight most common resin acids found in Canadian softwood pulp mill process waters.

Derivatized resin acids have been analyzed by high performance liquid chromatography (HPLC) (Richardson, et al. 1992). Direct analysis of dehydroabietic acid and abietic acid by HPLC is also possible (Kutney, et al. 1981; Lee, et al. 1997). An HPLC method for the direct analysis of dehydroabietic acid from the treatment lines of pulp mill effluent has also been developed (Chow and Shepard 1996). This method requires no hazardous chemicals during sample pretreatment and DHA concentrations as low 100 ng/mL are detected. Chow et al. (1996) obtained a good correlation of the DHA content determined by HPLC to the total resin acid content determined by a GC method. They were able to demonstrate that knowledge of the DHA content in effluent samples from a pulp mill will give the total resin acid content of those samples.

Immunoassay methods are often a good alternative to the traditional analytical methods such as HPLC and GC, GC/MS that are tedious and expensive to carry out routinely. In general immunoassays are simple, sensitive, cost effective and adaptable to laboratory or field situations and usually require little sample pretreatment before analysis. They have been successfully applied to a range of pesticides, (Hennion and Barcelo 1998) industrial chemicals (Eck, et al. 1990) and microbial toxins (Ramakrishna, et al. 1990). A DHA-conjugate that was used as an immunogen to produce polyclonal antibodies in rabbits was previously synthesized (Li, et al. 1994). These antibodies complexed DHA and significantly cross-reacted with other abietanes. They were used to develop a direct immunoassay based on ELISA that measured the amount of abietanes in pulp mill effluent samples (Li, et al. 1997). These workers showed abietane concentrations were a good marker for total resin acid content in pulp mill effluents. We used the same antibodies to develop an indirect competitive ELISA to measure dehydroabietic acid levels in the process waters of pulp and paper mills. However, the ELISA appears better suited as a semi-quantitative screening technique because of the high variability observed in the results.

RESULTS AND DISCUSSION

The polyclonal antibodies obtained by Li et al. (1994) were used to develop an ELISA to monitor DHA in pulp mill process streams. The antibodies were evaluated for their ability to complex free DHA in an indirect competitive ELISA. Unlike the assay developed by Li et al. (1994) the biotin-streptavidin system was not used. Removing this step made the assay faster, simpler and improved its selectivity and sensitivity towards DHA. The 50% inhibition concentration (IC_{50}) was determined to be 7 ± 2 ng/mL (ppb) for DHA. Characterization of the antibodies showed they cross-reacted with the abietanes: abietic and palustric acid, by 54% and 30% respectively (table 1). They did not, however, cross-react with levopimaric and neoabietic acid or the pimaranes tested.

TABLE 1. The Percentage Cross-Reactivity of the Anti-DHA-antiserum with other Resin Acids

Resin Acid	IC_{50} (ng/mL)[a]	% CR[b]
Dehydroabietic acid	7.0 ± 2.0	100
Abietic acid	13.0 ± 0.4	54
Palustric acid	23 ± 10	30
Levopimaric acid	91 ± 24	2
Neoabietic acid	6600 ± 2700	<1
Pimaric acid	490 ± 34	1.4
Isopimaric acid	690 ± 270	1.0
Sandaracopimaric acid	960 ± 200	<1

[a] All IC_{50} determinations were carried out in triplicate with standard deviations. [b] Percent cross-reactivity (%CR) for each resin acid, *x*, was calculated as $100[IC_{50}(x)/IC_{50}(DHA)]$

The significant cross-reactivity with other resin acids will make it difficult to use these antibodies to monitor a single resin acid amongst a mixture, however, they could be used to estimate the amount of abietanes present in the process waters. Mill process waters contain a variety of compounds such as wood lipids, phenolics, lignin, hemicellulose and cellulose. Representative compounds from these major classes were tested for their ability to inhibit antibody response (table 2). The compounds tested included fatty acids, fatty alcohols, sterols, steryl esters, waxes, mono-, di-, and triglycerides, lignins and glucomannans.

TABLE 2. Model Compounds of Products Possibly Present in Mill Process Waters that were Tested for Inhibition of Antibody Response

Class	Compounds Tested	IC_{50} (μg/mL)	% CR[a]
Fatty Acids	Myristic acid	> 64	< 1
	Palmitic acid	> 32	< 1
	Stearic acid	> 30	< 1
	Linoleic acid	> 30	< 1
Fatty Alcohols	Behenyl alcohol	> 50	< 1
Sterols	Stigmasterol	> 64	< 1
	β-Sitosterol	> 30	< 1
	Ergosterol	> 25	< 1
Steryl esters	Cholesteryl palmitate	> 12.5	< 1
Monoglycerides	1-Monostearoyl-rac-glycerol	> 250	< 1
	1-Monooleoyl-rac-glycerol	> 125	< 1
Diglycerides	Dipalmitin,	> 100	< 1
	Distearin	> 2	< 1
Trigylcerides	1,2-Dipalmitoyl-3-myristyl glycerol	> 6	< 1
Waxes	Palmitic acid oleyl ester	> 30	< 1
Lignin	Alkali	> 84	< 1
	Organsolv	> 60	< 1
	Catechol	> 60	< 1
Hemicellulose	Glucomannans	> 2.4	< 1

[a] Percent cross-reactivity (%CR) for each compound, *x*, was calculated as $100[IC_{50}(x)/IC_{50}(DHA)]$

In general cross-reactivity with these compounds was very low, however, antibody response was inhibited at high concentrations. Pulp mill process waters, however, are a complex matrix of many compounds both dissolved and undissolved of which only a few were tested. The ability of the immunoassay to detect DHA in either buffered solutions or resin acid-free newsprint process water sample obtained from the Millar Western Pulp Mill in Saskatchewan

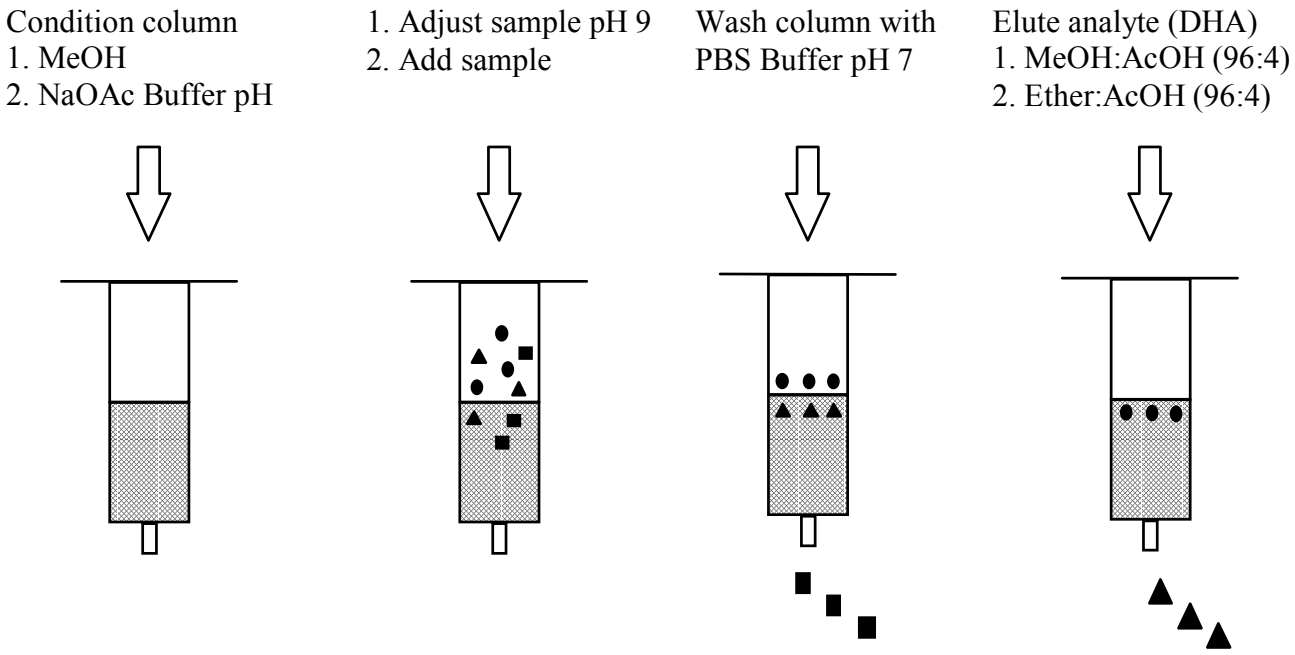
spiked with known amounts of DHA was tested. This mill uses a hardwood furnish to produce its pulp, therefore, the process waters are free of resin acids. The samples were used to simulate the matrix effects that may occur with resin acid containing process waters. Recovery results showed the immunoassay could measure DHA in buffered samples with percent recoveries ranging from 75-118%, however, recoveries from spiked process water samples were very poor and ranged from 130-250% (table 3). In general the assay significantly overestimated the amount of DHA in these samples. GC analysis of the process water samples verified they were free of other resin acids, however, it was clear components present in the process water adversely affected the immunoassay. It was obvious from these results that a pretreatment of the samples was necessary prior to the ELISA.

TABLE 3. Initial ELISA Results of DHA Spiked Newsprint Water Samples

Matrix	DHA Spiked (ng/mL)	Recovery by ELISA (ng/mL)[a]	(%)[a]
Buffer	5	3.8 ± 0.5	75% ± 11
	5	4.3 ± 0.5	86% ± 10
	5	5.9 ± 0.6	118% ± 16
NPW	5	12.4 ± 1.2	248% ± 36
	5	6.5 ± 0.6	130% ± 12
	5	11.5 ± 2.3	229% ± 46

[a] Recoveries were carried out in triplicate with standard deviations.

Various sample pretreatments such as filtration, centrifugation and dilution were used to remove the interfering components, however, none improved the immunoassay results. Only a rigorous sample pretreatment that included a liquid-liquid extraction, followed by a solid phase extraction on an aminopropyl column, resulted in acceptable ELISA recoveries. Once again, DHA spiked buffered and resin acid-free process water samples were used to test the effectiveness of the pretreatment. Recoveries for the buffered samples were 100 ± 14% while for the process water samples recoveries were 87 ± 18%. This pretreatment, however, is time consuming, generates significant amount of chemical waste and is not feasible for routine analysis of many samples. Therefore, a simple and effective sample pretreatment that eliminated the liquid-liquid extraction step was developed. Samples were purified with a solid phase extraction protocol using anion exchange. Complete DHA recovery as verified by HPLC, was achieved with the protocol outlined in scheme 1.



Scheme 1. A direct solid phase extraction procedure for purification of DHA from pulp mill process water samples.

The effect of DHA concentrations and sample loading volumes on recovery was also assessed (table 4). Excellent recoveries were obtained with DHA spiked phosphate buffer samples with loadings of up to 6 mL. However, recoveries were poor with loading volumes of 3 mL for DHA spiked mill process water samples. HPLC analyses confirmed DHA passed through during the buffer washing step. Nevertheless, this fast and simple pretreatment was effective in recovering DHA (2.5 to 30 μg/mL) from a 1 mL loading of the process water.

The solid phase extraction protocol was then tested on three resin acid-free pulp mill samples obtained at different process areas from the Millar Western Pulp Mill. The samples came from the twin roll pressate (TRP), interstage filtrate (ISF) and water recovery sump (WRS). The total methyl-*t*-butyl ether (MTBE) extractives content as well as the distribution of different lipophilic material of each sample were determined according to the protocol of Ekman et al. (1989). Total MTBE extractives were 0.24, 0.87 and 1.52 mg/mL for TRP, ISF and WRS respectively. The ISF and WRS samples had a similar distribution of steryl esters/waxes and triglycerides but significantly less sterols while these classes of compounds were found in similar amounts in the TRP sample. Interestingly these samples contained only trace amounts of fatty acids (fig. 2). The samples were spiked with known amounts of DHA then purified by solid phase extraction. Finally HPLC and ELISA were used to measure DHA recoveries.

Table 4. The Effect of Loading DHA Spiked Phosphate Buffered and Process Water Samples on an Aminopropyl Solid Phase Extraction Cartridge

Sample	DHA Concentration (μg/mL)	Sample Loading (mL)	HPLC Recovery (%)[a]
Phosphate Buffer	10	1	106 ± 2
	10	2	100 ± 1
	10	3	100 ± 2
	10	6	102 ± 3
Resin acid-free	2.5	1	108 ± 1
Process Water	5	1	105 ± 1
	10	1	99 ± 1
	20	1	96 ± 5
	30	1	94 ± 1
	10	3	39 ± 1

[a] Recoveries were carried out in triplicate with standard deviations.

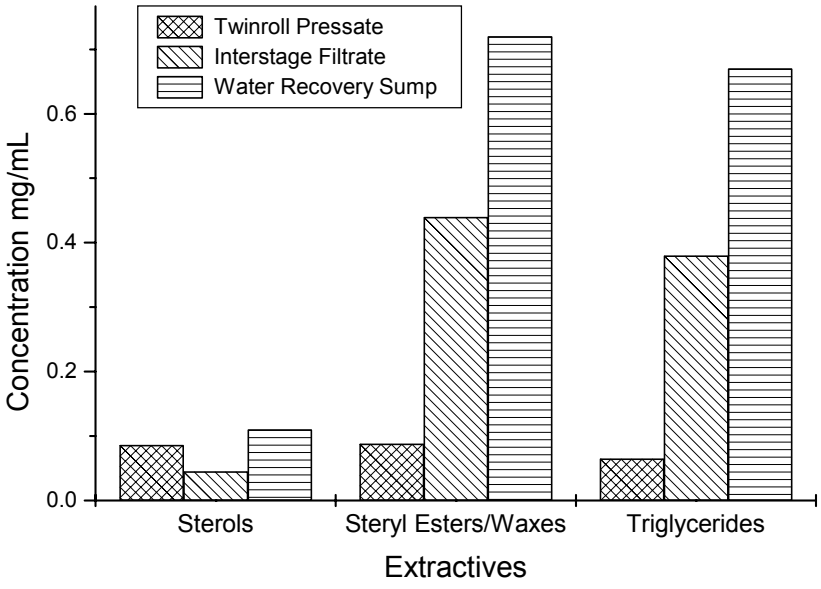


Figure 2. Distribution of lipophilic compounds in methyl-t-butyl ether extracts from resin acid-free pulp mill process water samples.

HPLC analysis using PeakFit software to compensate for baseline drifts, indicated that recoveries were essentially 100% (table 5). ELISA recoveries were near 100%, which is a marked improvement over recoveries determined with no pretreatment. This pretreatment worked for samples from different sites of the mill that contained different concentrations of extractives in varying proportions. However, there are several concerns that need to be addressed before this assay is applied to field situations. Large standard deviations were associated with the ELISA results that ranged between 6-58% with an average of 30%. The high variability suggests this assay is more useful as a screening method rather than a quantification tool. Since DHA and other resin acids are troublesome at concentrations higher than 1 mg/mL, this ELISA could be used to survey for "hot" samples because it can detect DHA at concentrations as low as 1 ng/mL. For such an application the industry must determine at which levels resins acids are problematic to their pulp and paper making processes.

TABLE 5. ELISA and HPLC Recoveries of DHA Spiked Pulp Mill Process Water Samples

Sample	DHA Concentration (μg/mL)	ELISA Recovery (%)[a] (2 ng/mL)[b]	(4 ng/mL)[b]	HPLC Recovery (%)[a]
TRP	5	84 ± 23	74 ± 28	105 ± 3
	10	94 ± 36	106 ± 30	103 ± 1
	20	119 ± 38	130 ± 52	96 ± 2
ISF	5	98 ± 22	136 ± 42	106 ± 7
	10	106 ± 16	127 ± 30	102 ± 2
	20	100 ± 6	94 ± 8	96 ± 3
WRS	5	104 ± 9	147 ± 58	112 ± 4
	10	152 ± 45	140 ± 30	104 ± 2
	20	88 ± 35	100 ± 33	96 ± 2

[a] Recoveries were carried out in triplicate with standard deviations. [b] Sample dilutions.

It is also necessary to clearly demonstrate that knowledge of the amount of abietanes determined by this immunoassay correlates to total resin acid content in process water samples. In other work it was demonstrated that DHA is a poor marker for total resin acid content of process water samples from an open TMP mill and that abietic acid concentrations were the best single marker (Serreqi, et al. 2000). It is known, however, that neoabietic, palustric and levopimaric acids isomerize to abietic acid under acidic conditions (Stoltes and Zinkel 1989), (Mutton 1962). These compounds eventually convert to DHA through an unspecified pathway (Quinde and Paszner 1991). In a closed mill, therefore, it is likely that the predominant resin acid will be DHA. If this is the case the immunoassay may be well suited for monitoring total resin acid content in process waters of closed pulp mills.

MANAGEMENT APPLICATIONS

The boreal forest of Canada is a vast resource of fibre for pulp and paper manufacturing. To reduce the impact of forest practices on this resource it must be managed carefully and efficiently. One aspect that can be controlled by closing the process water systems is the discharge from pulp and paper mills into the environment. Such measures, however, will require more rigorous monitoring of the process waters to manage the levels of dissolved and colloidal substances. Resin acids are an integral component of DCS that must be monitored. They are responsible for pitch deposition which adversely affects machine runnability and product quality. Developing fast, simple and reliable methods for monitoring such components will be essential for the Canadian pulp and paper industry to remain competitive in this toughening world market.

This study was carried out to assess the ability of an antibody based assay to monitor resin acids in pulp mill process. The results suggest the technology may be well suited as a screening method for samples with unacceptably high concentrations of these compounds. The method is easy to carry out, fast and does not require expensive analytical instruments. Also it is adaptable for on-site application. With further initiative it should be possible to transfer the technology from the laboratory to the mill.

CONCLUSIONS

An ELISA was developed to monitor DHA in buffered water samples. The assay is very sensitive with an IC_{50} of 7 ± 2 ng/mL. The antibodies are very selective for DHA and some DHA-like resin acids. However, when the assay was used to determine recoveries of DHA spiked pulp mill process water samples, unidentified components adversely affected the assay. A simple solid phase extraction was developed to remove the interfering components prior to analysis. The pretreatment was tested on resin acid-free pulp mill process water samples from different sites of the mill with varying extractive concentrations. The samples were spiked with known amounts of DHA and good recoveries were obtained as analyzed by ELISA and HPLC. Large standard deviations, however, were associated with the ELISA results that ranged between 6-58% with an average of 30%. This ELISA has potential as a screening tool for concentrated DHA samples because of its high sensitivity; however, quantitative analysis will be difficult because of the great variability of the results.

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