Recovery and Purification of 10-oxo-*trans*-8-decenoic Acid Enzymatically Produced Using a Crude Homogenate of *Agaricus bisporus*

RUBEN O. MORAWICKI, ROBERT B. BEELMAN, AND DEVIN PETERSON

ABSTRACT: A two-step method was developed to recover 10-oxo-*trans*-8-decenoic acid (ODA) at 85% purity using GRAS solvents that may be used either at laboratory or industrial scale. ODA was recovered from an aqueous reaction broth via extraction with ethyl acetate followed by evaporation. The residue was dissolved in hot hexane and subsequently crystallized at 5 °C. Optimal recovery of ODA from the reaction broth was optimized by determining the partition coefficient between phosphate buffer (range pH 2.0 to 7.5) and ethyl acetate. The intrinsic partition coefficients were 75.38 and 1.43 for the undissociated and dissociated forms, respectively. To obtain a good recovery, the optimal pH was determined to be 3.0. Purification was optimized by determining the solubility curve of ODA in hexane as a function of temperature. The solubility of ODA in hexane decreased from 0.7 mg/mL at 50 °C to 0.05 mg/mL at 10 °C. The solubility at intermediate temperatures followed a linear van't Hoff model, indicating an approximately constant enthalpy of solution. Even when the solubility of ODA in hexane is relatively low, the temperature-solubility profile was adequate to recrystallize ODA.

Keywords: 10-oxo-*trans*-8-decenoic acid, van't Hoff equation, partition coefficient, recrystallization, purification

Introduction

10-Oxo-*trans*-8-decenoic acid (ODA) is a secondary metabolite of many mushroom species. ODA comes from the enzymatic breakdown of linoleic acid apparently mediated by a lipoxygenase and a hydroperoxide lyase (Wurzenberger and Grosch 1982). Wurzenberger and Grosch (1982) first suggested that ODA could be involved in some growth regulation mechanism like its homologue,12-oxo-*trans*-10-dodecenoic acid, is in green plants. Furthermore, Mau and others (1992) reported that ODA produced elongation on separated stipes of *Agaricus bisporus* and also increased the linear growth of mycelium on potato dextrose yeast agar. They also indicated that supplementation of the casing layer with ODA resulted in higher yields of the 1st flush of *Agaricus bisporus* crops. Champavier and others (2000) replicated Mau's experiments and confirmed that ODA produced stipe elongation. However, they observed only a marginal increase of mycelia growth compared with the results reported by Mau and others (1992).

In addition to its in vivo role in mushrooms, ODA may also have a practical application as a natural antimicrobial or fungal growth enhancer. Comes (2001) found that ODA produced a 3-log reduction in the count of *Escherichia coli O157: H7* in apple cider. Okull (2002) demonstrated the efficacy of undissociated ODA at inhibiting the mycelial growth of *Penicillium expansum*. On the other hand, a recent U.S. patent reported that ODA stimulates the fungal growth and production of secondary metabolites in submerged cultures (Farbood and others 2001). Beelman and others (2003) indicated that ODA in the dissociated form is stimulatory for the growth of fungi.

Even though some progress has been made in the last 20 y regarding the in vivo role and applications of ODA, research has moved slowly, probably because of a lack of an abundant and cheap source of ODA. Therefore, a method for its rapid recovery and purification would be desirable to continue the research in this area.

Tressl and others (1982) and Wurzenberger and Grosch (1982) isolated ODA for the 1st time as a methyl ester. Then, Mau and others (1992) and Champavier and others (2000) isolated and purified this compound, as a free acid, using extraction with ethyl ether followed by column chromatography and thin-layer chromatography. However, the latter method is laborious and only practical to produce small amounts of ODA. Also, the use of ethyl ether is not acceptable for the potential use of ODA in foods.

The development of a new method to avoid the chromatographic procedures would be very convenient to produce ODA. A viable alternative to the purification procedure is recrystallization. This technique has been extensively used to purify many molecules of natural origin (Kennedy and Cabral 1993; Cannell 1998; Walton and Brown 1999).

The objective of this paper was to develop a method to recover and purify ODA from a reaction broth using liquid-liquid extraction and crystallization utilizing the GRAS solvents ethyl acetate and hexane. Partition coefficients of ODA in a phosphate buffer/ethyl acetate system, and the solubility of ODA in hexane were studied to obtain the optimal parameters for the recovery and crystallization, respectively.

ODA was produced by blending mushroom tissue in an appropriate buffer with the addition of linoleic acid and oxygen. For this research, fruiting bodies of *Agaricus bisporus* were used because the ultimate objective of the research project was to find alternative applications for some of the byproducts of the mushroom industry (Morawicki and others 2005).

During harvesting, mushrooms are picked from the substrate and the end tip of the stems ("stumps") are removed and discarded. These stumps are an important byproduct for the mushroom producers. In addition, around 10% of the harvested mushrooms are 2nd grade quality with low commercial value. Therefore, these 2 byproducts have potential as a source of enzymes to produce ODA.

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Materials and Methods

Materials

1-Octen-3-ol (98%), 2-heptanone (98%), *cis*-pinonic acid (98%), and linoleic acid (99%) were purchased from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). 10-Oxo-*trans*-8-decenoic acid (ODA), used as analytical standard, was provided by Centre Ingredient Technology, Inc. (State College, Pa., U.S.A.). Purity of ODA was assessed by mass spectroscopy and used without further purification. Fresh mushrooms (*Agaricus bisporus*) were obtained from the Mushroom Test and Demonstration Facility at The Pennsylvania State Univ. (Univ. Park, Pa., U.S.A.) Mushrooms were harvested and refrigerated at 4 °C within 30 min after harvesting.

Enzymatic production of ODA

ODA was prepared enzymatically by blending 334 g of fresh *Agaricus bisporus* mushrooms for 1.0 min in 666 mL 0.1 M phosphate buffer, pH 7.5, with the initial addition of 4.0 g of 99% pure linoleic acid. After blending, the homogenate was transferred to a 1-L bioreactor and the reaction run at 8 °C under constant stirring and oxygen bubbling. Dissolved oxygen was monitored with an ORION Sensor Link™ PCM800 dissolved oxygen measurement system (ORION Research Inc., Beverly, Mass., U.S.A.) connected to an IBM computer used for data acquisition. Calibration of the probe was done with deionized water saturated with air at 25 °C following the method suggested by the manufacturer. No correction was made for the presence of solids in the reaction broth. Multiple batches were run when necessary. A more detailed description of the method has been published by Morawicki and others (2005).

Recovery and purification

Recovery of ODA from the reaction broth followed the general strategy presented in Figure 1. After the reaction was completed, pH was adjusted to 3.0 to promote full protonation of ODA, and then the acidified broth was extracted with ethyl acetate. Solvent was then evaporated in a rotary evaporator, the residue recovered, and ODA purified by crystallization in hexane. The parameters pH for liquid-liquid extraction and temperature for crystallization were established by determining the ODA partition coefficients between ethyl acetate–water and the solubility of ODA in hexane as a function of the temperature.

Partition coefficients

Partition coefficients of ODA as a function of pH were determined with a modified version of the method described by Streng (2001). Experiments were conducted in duplicate using 2 different initial concentrations of ODA. Due to its acid nature, ODA's partition coefficient is a function of pH. Therefore, 0.01 M phosphate buffer solutions were used ranging from pH 1.7 to 8.0 at intervals of approximately 1 unit. Buffers were saturated with ethyl acetate by adding approximately 10% by volume of ethyl acetate and shaking vigorously for 5 h. Then the phases were allowed to settle, and the solvent was recovered. Similarly, the organic phase was saturated with water following the same procedure.

The experiment was conducted by adding 20 mL ethyl acetate-saturated buffer and 20 mL water-saturated ethyl acetate, containing ODA, to a 100-mL conical flask for each pH. The flasks were then placed in a temperature controlled water bath with agitation. Temperature was set at 24 °C, and the samples were agitated at a speed just enough to disturb the interface until equilibrium was reached. To establish equilibration time, samples of 1 μL of the organic phase were withdrawn from selected flasks and analyzed for ODA. Equilibration time was established as the one that produced no further change in the concentration of the organic phase.

Once equilibrium was reached, both the organic and the aqueous phases were analyzed using gas chromatography to quantify the concentration of ODA. To 1 mL of the ethyl acetate phase, 0.45 mg of the internal standard *cis*-pinonic acid was added and from that a 1 μL injection was made into a GC. Samples of the aqueous phase were acidified to pH 2.0 and extracted twice with ethyl acetate. The 2 extracts were pooled and then analyzed for ODA.

Partition coefficients (Pow = [ODA]organic phase/[ODA]aqueous phase) were calculated as the ratio of the concentration of ODA in the organic to ODA in the aqueous phase. The experimental data was then fitted, using nonlinear regression, to the model given by Eq. 1 using the minimization of the least squares:

$$Pow = \frac{k_o + k_1\left(\frac{K}{[H]}\right)}{1+\left(\frac{K}{[H]}\right)} \quad (1)$$

where Pow = observed partition coefficient, k_i = intrinsic partition coefficients, K = dissociation constant = 0.00002, and $[H]$ = concentration of protons.

Solubility of ODA in hexane

Ten milligrams ODA and 1 mL hexane were placed in 5 vials, which were then tightly capped and sonicated for 30 min at 60 °C. Immediately, each vial was placed in water baths at 50, 40, 30, 20, and 10 °C and held until a clear solution was observed (approximately 24 h). Samples of the clear supernatant were withdrawn with a pipette, diluted with ethyl acetate, and after addition of *cis*-pinonic acid (the internal standard) analyzed for ODA content. This experiment was conducted in triplicate.

Experimental data were fitted to both linear (Eq. 3) and nonlinear

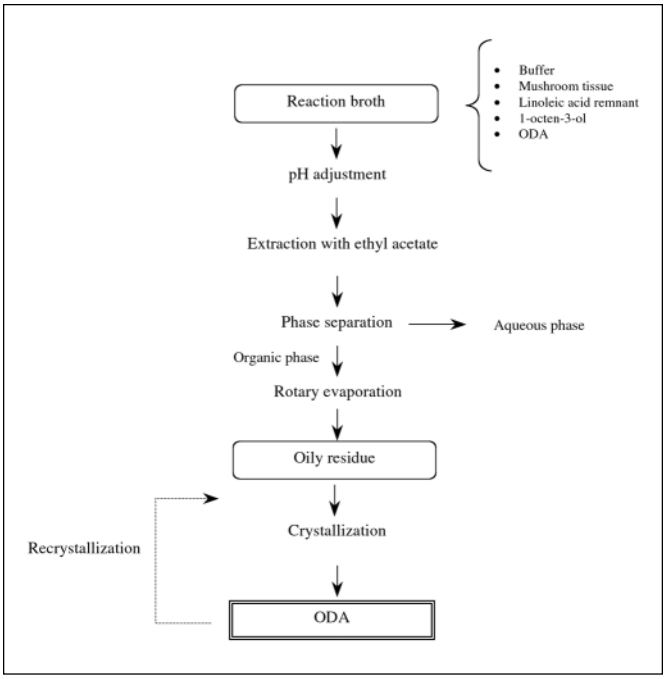

Figure 1—General procedure for recovery and purification of ODA from a reaction broth

(Eq. 4) van't-Hoff type equations. For the linear model, enthalpy of solution was assumed not to vary with the temperature within the studied range:

$$\text{van't Hoff: } \frac{d\ln S}{dT} = \frac{\Delta H_S}{RT^2} \quad (2)$$

$$\text{linear: } \ln(S) = -\frac{\Delta H_S}{R}\frac{1}{T} + b \text{ (Krug and others 1976)} \quad (3)$$

$$\text{nonlinear: } \ln(S) = -\frac{a}{R}\frac{1}{T} + \frac{b}{R}\ln T + c \text{ (Grant and others 1988)} \quad (4)$$

where S = solubility in mg/mL, T = absolute temperature in Kelvin, ΔHs = enthalpy of solution of the solute, R = gas constant, and a, b, and c = constants.

Analytical procedures

The organic solvents containing the analytes were injected onto the split/splitless injector of a Hewlett Packard 5890A gas chromatograph equipped with a FID detector and a 30 m, 0.32 Idado fused silica capillary column Supelco SPB-5, 0.25 μm film thickness (Supelco, Bellefonte, Pa., U.S.A.). Both injector and detector were maintained at 250 °C.

When required, 1-octen-3-ol and ODA were analyzed simultaneously using 2-heptanone and *cis*-pinonic acid as internal standards, respectively. The oven temperature was held at 73 °C for 5 min, then ramped to 180 °C at 40 °C/min, and maintained 180 °C for 7 min. When quantification of 1-octen-3-ol was not necessary, ODA was analyzed isothermally with the oven at 180 °C.

Confirmation of the purity

Purity of the final product was determined with GC and mass spectroscopy (MS) analysis. The purified material was dissolved in ethyl acetate (0.5 mg/mL) and injected into a GC following the procedure described above. The result obtained with GC was further confirmed by MS analysis utilizing electrospray ionization analysis (negative anion mode). Mass spectroscopy analysis was conducted at The Pennsylvania State Univ.'s Dept. of Chemistry.

Results and Discussion

Partition coefficients

Like any other weak acid, ODA's protonated form is neutral and the dissociated (nonprotonated) form is negatively charged. Our partition experiments reported that as the pH ranged from 1.5 to 3.0, most of the ODA was partitioned in the organic phase (Figure 2). In contrast, at pH values above 3.0, most of the ODA was solubilized in the aqueous phase. As anticipated, the undissociated form of ODA is relatively hydrophobic while the dissociated form is hydrophilic.

Table 1—Intrinsic partition coefficient for ODA between ethyl acetate and 0.01 M phosphate buffer

Intrinsic partition coefficients	Value
k_0 (uncharged)	75.38
k_1 (charged)	1.43

Intrinsic partition coefficients were calculated by fitting Eq. 1 with nonlinear regression methods using a pKa of 4.68 (Mau and others 1992). The fit of the mathematical model to the experimental data was confirmed by the high correlation coefficient (R^2 = 0.972). As a result of the regression, intrinsic partition coefficients were obtained for the protonated and unprotonated forms of ODA (Table 1).

Solubility of ODA in hexane

The temperature dependence of ODA solubility in hexane was studied between 10 °C and 50 °C. Experimental results showed that the solubility increased about 7-fold as the temperature was raised from 10 °C to 50 °C. Data of solubility as a function of temperature were fitted to both linear and nonlinear van't Hoff models. The proximity of the sum of the residual squares, 0.055 and 0.077 for the linear and nonlinear equations (Table 2), respectively, showed that the dissolution enthalpy of ODA does not vary considerably in the range of temperatures studied. Therefore, the dissolution enthalpy of ODA in hexane can be assumed to have a constant value of 285 J/g. Figure 3 is an illustration of the good agreement between experimental data and the linear regression line. Calculations of the dissolution enthalpy were conducted using the parameters and the linear equation presented in Table 3.

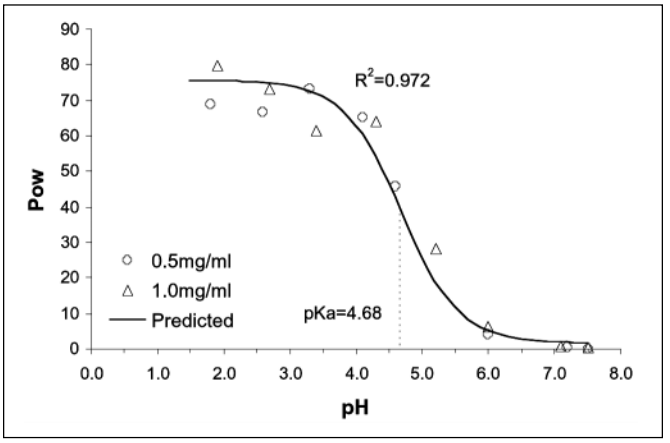

Figure 2—Partition coefficients (Pow) of ODA between ethyl acetate and 0.01 M phosphate buffer determined at 25 °C. Predicted values are the best fit of Eq. 1 using the minimization least squares.

Table 2—Fitting of linear and nonlinear van't Hoff model for solubility of ODA in hexane as a function of temperature

					Nonlinear van't Hoff		Linear van't Hoff	
Temp (°C)	Average solubility (mg/mL)	STD	1/T (1/K)	ln (solubility)	Estimated ln (solubility)	Residual squared	Estimated ln (solubility)	Residual squared
50.0	0.699	0.0191	0.0031	–0.3584	–0.2319	0.0160	–0.2528	0.0111
40.0	0.432	0.0276	0.0032	–0.8403	–0.88 88	0.0023	–0.8779	0.0014
30.0	0.257	0.0172	0.0033	–1.3578	–1.5670	0.0437	–1.5441	0.0347
20.0	0.096	0.0076	0.0034	–2.3387	–2.2679	0.0050	–2.2559	0.0069
10.0	0.047	0.0015	0.0035	–3.0534	–2.9932	0.0036	–3.0179	0.0013
Sum of residual squared						0.0707		0.0554

Even when the yield was low, the solubility-temperature curve for ODA in hexane (Figure 3) suggested hexane to be a good solvent to purify ODA by recrystallization. Impure ODA was dissolved in hexane at 50 °C and then the temperature reduced to below 10 °C to promote crystallization. Much lower temperatures (such as –20 °C) accelerated crystallization. However, the purity of the final product was compromised as many impurities coprecipitated with or became entrapped inside the ODA crystals (data not shown). On the other hand, temperatures around 5 °C produced a slower crystallization that took up to 12 h, which resulted in improved purity of ODA.

Recovery of ODA from the reaction medium

According to preliminary assays, the mixture of ethyl acetate and the reaction broth produces stable emulsions that require high amounts of energy to break.

Therefore, to minimize the emulsification, the broth was initially clarified to partially eliminate tissue particles that help to stabilize the emulsion.

Figure 4 presents a flowchart for the recovery process with a clarification step. Initially, 2000 g of the reaction broth was prepared using 99% pure linoleic acid, and divided into 2 batches of 1000 g each. Then the whole procedure was conducted for duplicate analysis of each batch.

The broth (1,000 g) containing essentially buffer, mushroom tissue, 674 mg 1-octen-3-ol and 950 mg ODA was acidified to pH 3.0 and centrifuged to separate the coarse solids. This separation generated Supernatant I and Pellet I.

Supernatant I was clarified by adding 4.0 g $Fe_2(SO_4)_3$ followed by a settling period and centrifugation. After centrifugation, Supernatant I generated Supernatant II and Pellet II. Supernatant II was extracted twice with ethyl acetate. The 1st extraction (Fraction 1) recovered 74% of the total ODA obtained with this procedure. The 2nd extraction (Fraction 2) recovered only 5.4%. Pellet II was washed with solvent and 5% of ODA was recovered. Clearly, a 2nd extraction with solvent for Supernatant II and the recovery of ODA from Pellet II were not significant.

Pellet I was washed twice with 200 mL fresh ethyl acetate. Approximately 12% of the ODA was recovered in the 1st wash (Fraction 4) and 4.2% in the 2nd wash (Fraction 5).

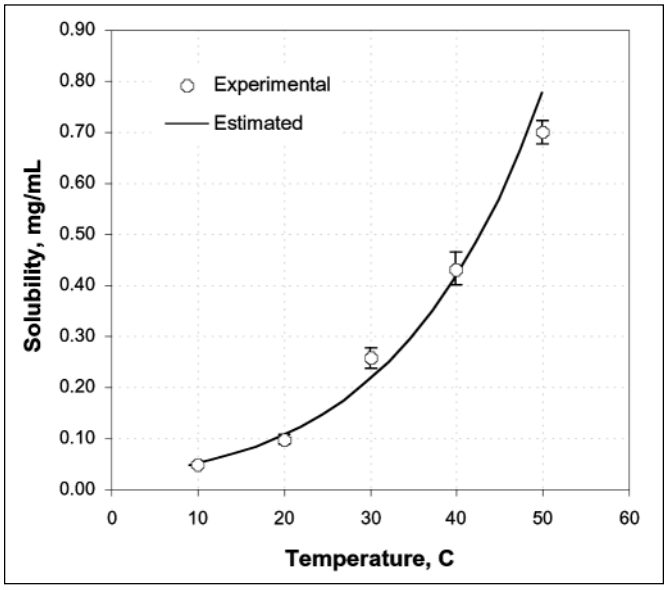

Figure 3—Solubility of ODA in hexane as a function of the temperature. Experimental values are the average of 3 replicates. Estimated values (solid curve) were obtained with a linear van't Hoff model.

Table 3—Parameters for the linear and nonlinear van't Hoff model for solubility of ODA in hexane as a function of temperature

Model	Equation	a	b	c
Linear	ln S = a/T +b	–6318.8	19.31	—
Nonlinear	ln S = a/T +b lnT+c	2.75821	20.8773	–120.84557

Table 4—Material balance for the recovery procedure presented in Figure 5[a]

Description	Mass (g)	Volume (mL)	$m_{1\text{-octen-3-ol}}$ (mg)	m_{ODA} (mg)
Broth	2000		674 ± 50	950 ± 35
Supernatant I	801.3 ± 3.6		—	—
Pellet I	198.6 ± 3.7		—	—
Supernatant II	766.1 ± 2.8		—	—
Pellet II	36.5 ± 2.6		—	—
Fraction 1	—	732.5 ± 103	300 ± 28	560 ± 42
Fraction 2	—	755.0 ± 9.8	48 ± 6	41 ± 7
Fraction 3	—	69.5 ± 10.8	47 ± 14	34 ± 7
Fraction 4	—	119 ± 41.2	87 ± 3	89 ± 6
Fraction 5	—	153.5 ± 6.9	41 ± 20	32 ± 2

[a]Milligrams of 1-octen-3-ol and ODA were calculated after quantification of the analytes by gas chromatography.

By performing only the steps conducting to Fraction 1 and Fraction 4, 86% of the final ODA was recovered. Therefore, from a practical viewpoint, the steps that lead to Fractions 2, 3, and 5 could be eliminated.

On average, 79% of the ODA that was initially contained in the broth was recovered with the strategy presented in Figure 4. The rest was lost in the discarded aqueous phases (Table 5).

Purification of ODA by crystallization in hexane

The solubility-temperature curve obtained in the previous section was used to design a method to purify and crystallize ODA contained in the captured material (Figure 4). Fractions 1 to 5 were pooled and evaporated at 50 °C in a rotary evaporator under vacuum (Figure 5). The residue after evaporation had a waxy consistency with a strong smell of 1-octen-3-ol. This residue was washed 8 times with 10-mL fractions of hexane at 50 °C. These fractions were then cooled to 5 °C, and after 24 h, crystals were recovered. Following harvesting, the crystals were rinsed with cold hexane (5 °C) and dried under a current of N_2. The final weight

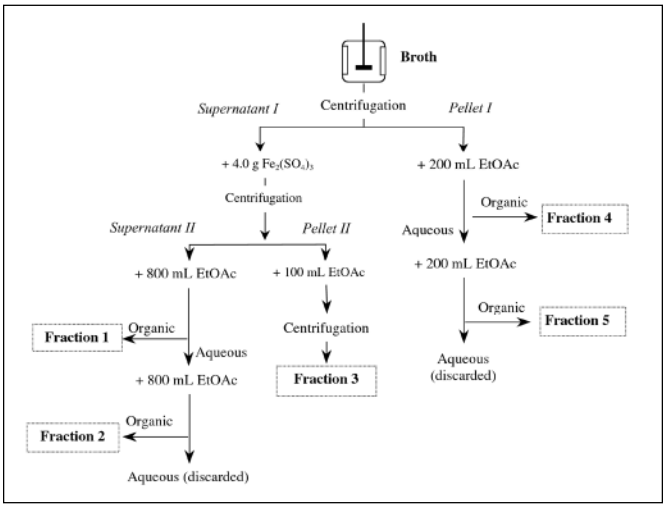

Figure 4—Recovery of ODA from the reaction broth using ethyl acetate (EtOAc) with a clarification step. ODA was enzymatically prepared using 99% pure linoleic acid. The mass balance is presented in Tables 4 and 5.

of the crystals was 642 ± 12 mg. Quantitative GC analysis of the crystalline material was determined to consist of 85% ODA. The high purity of ODA was also supported by mass spectroscopy analysis, which suggested that ODA was the chemical species with the highest concentration (Figure 6). The base peak of the mass spectrum is ODA $[M-1]^{-1}$ with an m/z of 183.0, and the peak with an m/z of 201.0 is monohydrated ODA.

One of the main impurities was the diacid form of ODA, *trans*-2-decen-1, 10-dioic acid. In the mass spectrum, the diacid is the peak with an m/z = 199.0. From the mass spectrum, the suspected diacid has approximately 10% of the intensity of ODA. The diacid could have been formed either during the reaction or during the purification. Recrystallization did not produce purer ODA, thus suggesting that the solubility-temperature profiles for ODA and the diacid are probably very similar.

After crystallization of ODA, hexane fractions were pooled and evaporated. The residue was 2.7 g of an oily residue of which the main compound was 1-octen-3-ol.

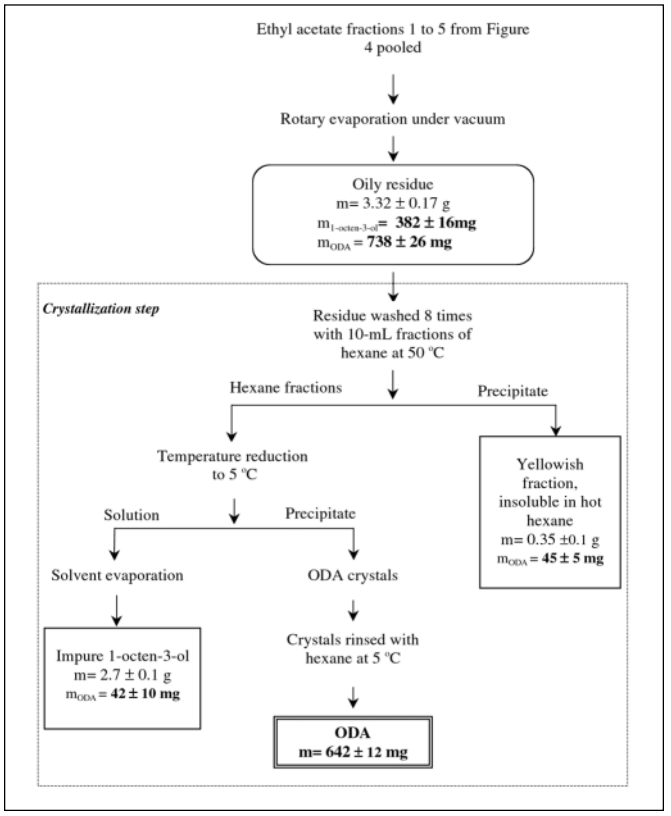

Figure 5—Purification of ODA prepared enzymatically with 99% pure linoleic acid. Values are the results of duplicates.

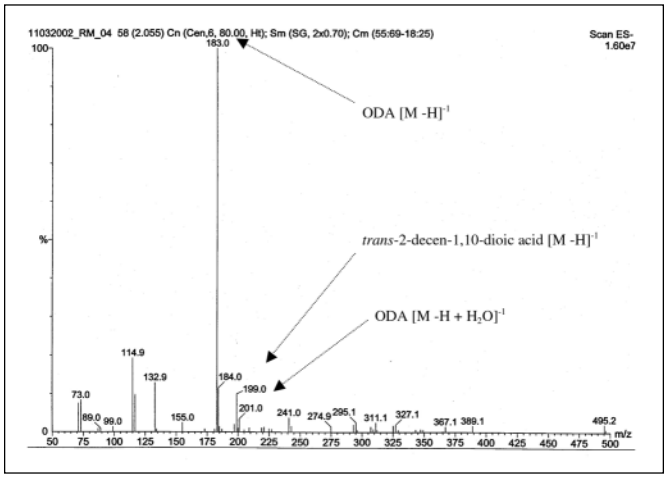

Figure 6—Electrospray ionization mass spectrum analysis (negative anion mode) of crystallized ODA prepared with 99% pure linoleic acid.

Table 5—Mass balance of the extraction process of ODA from the reaction broth using ethyl acetate (Figure 5)

	EoOAc (mL)	1-octen-3-ol (mg)	ODA (mg)
Initial	2000	674	950
Recovered	1829	523	756
Lost	171	194	151
Recovery, %	91	79	77

Conclusions

ODA was recovered using liquid-liquid extraction with ethyl acetate and subsequently purified by crystallization in hexane. These methods can be used either at lab-scale or be scaled-up to industrial production levels. To the best of our knowledge, this is the 1st time ODA has been crystallized from a solvent. All previous methods found in the literature involved extraction with ethyl ether and 2 chromatographic steps: a silica column, and thin-layer chromatography. However, the purity of ODA obtained with our method was slightly lower than the ones obtained for the previous procedures. The main contaminant is apparently the diacid form of ODA (*trans*-2-decen-1, 10-dioic acid), therefore further studies are needed to minimize the formation of this compound either during the reaction or the purification procedure.

Acknowledgments

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