IMPROVED STABILIZATION OF 2-ACETYL-1-PYRROLINE ZINC CHLORIDE COMPLEX USING SPRAY CHILLING ENCAPSULATION AND ITS PRACTICAL APPLICATION IN FOODS

BY

YUN YIN

DISSERTATION

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Doctoral Committee:

Professor Hao Feng, Chair
Professor Keith R. Cadwallader, Director of Research
Assistant Professor Youngsoo Lee
Teaching Assistant Professor Dawn Bohn

Abstract

2-Acetyl-1-pyrroline (2AP) is an important and characteristic odorant in aromatic rice and a multitude of other foods. Despite its desirable aroma attributes, the great instability of 2AP has hindered its widespread commercial use by the flavor industry. Various approaches have been attempted to increase its stability; however, none of them showed potential for real applications in foods. Our lab pioneered a stabilization method whereby 2AP is complexed with a zinc halide. This complexed form of 2AP maintains excellent stability under anhydrous conditions. However, it degrades rapidly when moisture is present.

The overall objective of this study was to improve the stability of 2AP zinc halide complex by use of spray-chilling encapsulation, and to demonstrate the practical application of the stabilized microcapsules as flavoring agents in foods. First, the use of 2AP-$ZnCl_2$ complex itself as a flavoring agent in instant rice was evaluated and high flavor recovery of 2AP (92%) was found in the final product. Secondly, the feasibility of encapsulating 2AP-$ZnCl_2$ by a modified spray-chilling encapsulation process using paraffin wax (octacosane) was achieved. The physical properties of the microcapsules were measured by scanning electron microscopy (SEM) and X-ray micro-computed tomography (CT). The microcapsules were shown to have matrix type structure and possess other preferred characteristics. The chemical properties were measured by gas chromatography (GC) and by absorbance spectroscopy, whereby the 2AP retention after spray chilling process was shown to be 65.26%. Additionally, the 2AP stability in both microcapsule and unprotected complex forms were monitored under ambient temperature (at 0%, 22.5%, or 43.2% relative humidity) conditions for more than three months. The results demonstrated significantly enhanced stability in the microcapsule form under all storage

conditions. Finally, the controlled release and flavoring properties of the 2AP-$ZnCl_2$ microcapsules were verified by full flavor recovery after cooking in instant rice. The results showed that the loss of 2AP in microcapsule form during cooking was negligible (not statistically significant). Overall, the aims of the study were achieved and the application of the 2AP-$ZnCl_2$ microcapsules as a flavoring agent in foods was examined to be feasible.

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Chapter 1

INTRODUCTION

1.1 Background

2-Acetyl-1-pyrroline (2AP) is an aroma compound that possesses a roasty, popcorn or scented rice-like note. It was first identified in cooked rice[1] and is an important aroma component of many foods.[2–8] It can be formed biochemically in aromatic rice[9] and in pandan leaves[10]. However, its wide occurrence in most foods can be attributed to its formation via the Maillard reaction; it is well accepted that 1-pyrroline formed from proline via the Strecker degradation is the key intermediate involved in 2AP formation.[11] Additionally, some *Bacillus cereus* strains have been shown to produce 2AP from proline, glutamic acid and glucose.[12]

2AP is of potential commercial interest to the flavor industry because it possesses unique and pleasant aroma attributes and because of its wide occurrence in foods. It is also a powerful odorant due to its low odor detection threshold of 0.1 parts-per-billion in water.[13] However, its great instability has hindered its commercial application by the flavor industry.

Many efforts have been taken to increase the stability of 2AP by various chemical and physical approaches. While these studies improved the stability of 2AP to some extent, all suffered from at least one of the following deficiencies: 1) stability of 2AP was either not mentioned or was insufficiently studied; 2) low loadings of 2AP were used; and 3) 2AP was only conditionally stable at low temperature (4°C and -20°C) storage.[14]

Our lab has developed a promising stabilization method, whereby 2AP is coordinated as a ligand onto a zinc halide forming a stable powdered complex with excellent 2AP stability when stored in a dry environment. Free 2AP can be released upon hydration of the complex. The stability of 2AP in ZnI_2 complex was measured over a period of 3 months' storage at three different temperatures (-20°C, 10°C and 25°C), a 94% retention was observed for 25°C storage.[14–16] This study was the first time 2AP showed almost no degradation when stored under ambient temperature conditions. However, the complex degraded immediately when moisture was present; therefore, a hydrophobic layer is required to coat the complex and protect it from moisture invasion.

1.2 Overall hypothesis and goals

It is hypothesized that wax encapsulated 2AP zinc halide complexes can be effectively used as flavoring agents in foods provided: 1) they are appropriately protected from moisture during handling and storage, and 2) they can undergo controlled flavor release when the microcapsules are heated above the melting point of the wax, thus allowing moisture to react with the complex with a concurrent release of 2AP.

Therefore, the corresponding **long term goals** of this study include: 1) Improving the stability of 2AP zinc halide complex by microencapsulation with a wax coating; 2) Enabling the use of these stabilized microcapsules as flavoring agents in real food matrices.

1.3 Specific Objectives

The following specific objectives were completed in this study:

1). Evaluating the potential use of the 2AP-$ZnCl_2$ complex as a flavoring agent in instant rice

2AP-$ZnCl_2$ was chosen because of its Generally Recognized as Safe (GRAS) substances. The complex was expected to impart an aromatic flavor to bland rice after cooking since the 2AP molecule could be freed from the complex upon hydration. The feasibility of using the complex to improve the flavor profile for instant rice was investigated, the high recovery of 2AP (92%) was achieved after the cooking process, and its application potential as a flavoring agent was therefore considered to be promising.

2). Feasibility study of encapsulating 2AP-$ZnCl_2$ complex by spray-chilling technique using wax

Although the complex was proved to have the potential of being an excellent flavoring agent, it is readily degraded at ambient conditions due to moisture invasion. However, the stability of the complex could be retained if it is coated with a hydrophobic layer with low water vapor permeability using an appropriate encapsulation technique. In this objective, the 2AP-$ZnCl_2$ complex was successfully coated with a paraffin wax (octacosane) layer by spray chilling encapsulation and free flowing microcapsules with high flavor retention (65.26%) of 2AP were achieved. Meanwhile, the spray-chilling apparatus was modified, appropriate wax materials were selected and targeted aroma complexes were identified to facilitate the process.

3). Chemical and physical characterization of 2AP-$ZnCl_2$ microcapsules

Physical and chemical methods are required to characterize 2AP-$ZnCl_2$ microcapsules in order to improve its functionality and allow for future modification. Testing tools including scanning electron microscopy (SEM) and X-ray micro-computed tomography (CT) were applied to investigate the physical properties of microcapsules. Meanwhile, appropriate methods for determining 2AP and $ZnCl_2$ contents in microcapsules were developed by use of gas chromatography (GC) and absorbance spectroscopy measurement, respectively. The results demonstrated that the microcapsules possessed desirable characteristics for real food application.

4). Investigation of the 2AP stability in microcapsules during storage at ambient conditions

The 2AP stability in microcapsules can be determined by use of a time-course study by monitoring the changes in 2AP content. Three relative humidity (RH) conditions (~0%, ~22.5% and ~43.2%) at ambient temperature were chosen for storage studies on both microcapsules and the unprotected complex. The results showed that the microcapsules retained significantly improved 2AP stability compared to the unprotected complex at all storage conditions. The microcapsules were shown to have the potential for commercialization due to the high 2AP stability for more than 3 months, as long as they are packaged in a moisture-insulated bag.

5). Application of 2AP-$ZnCl_2$ microcapsules as a controlled release flavoring agent in foods

The 2AP-$ZnCl_2$ complex remained stable in the microcapsules during blending and handling practices since the wax is an effective moisture barrier to protect the complex from

degradation. The wax matrix will be melted during cooking causing the complex to be exposed to moisture thus resulting in the release of the free 2AP molecule. The practicability of using the microcapsules as an effective flavoring agent was validated, by achieving high flavor recovery (almost 100%) during the cooking process of instant rice. Comparing to the pure complex, the microcapsules form was verified to have several advantages including possessing the controlled release property and high 2AP recovery during application, as well as requiring easier handling and transfer practices.

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Chapter 2

LITERATURE REVIEW

2.1 2-Acetyl-1-pyrroline and its importance in foods

2-Acetyl-1-pyrroline (2AP) is an aroma compound which exhibits a roasty, popcorn or cracker-like note. It was first identified in cooked rice[1] and later in corn products[2]. Its structure is shown in Figure 2.1. 2AP is an important aroma contributor to the flavor of a multitude of foods, especially it provides the characteristic note of aromatic/scented rice. Although 2AP concentrations in foods are generally low in the parts-per-billion range (Table 2.1), it is still a powerful odorant due to its extremely low odor detection threshold of 0.1 parts-per-billion in water.[3] 2AP is of potential commercial interest to the flavor industry because of its unique and pleasant aroma attributes and potential for wide application in foods. However, the commercial use of 2AP has been hindered because of its great instability.

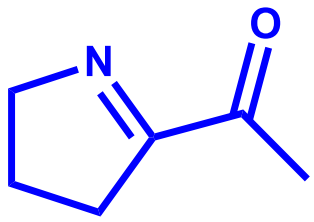

Figure 2.1 The structure of 2AP.

Table 2.1 2AP concentrations in various foods generally exceed its odor detection threshold of 0.1 ppb.

Food category	Food products	Measured 2AP concentration (ppb)	References
Staple foods	Cooked rice	6-90	4
	California long grain rice	0.6	3
	Aromatic rice	76-156	5
	Rice	10-1104	6
	Rice	0-1555	7
	Cooked black rice	0.3-1.7	8
Plants	Bread Flowers (*Vallaris glabra* Ktze)	0.53-26.12	9
	Pandanus amaryllifolius Roxb	90-4980	10
Value-added foods	Rice cake	10-21	11
	Corn Tortillas	1-10	12
	Popcorn	6-57	13
	Sweet corn products	2-44	2
	Wheat and rye bread crusts	0-78	14
	Pecans	80-146	15
	Peanut meal	0.1-11	16
Aquatic Products	Cooked Spiny Lobster Tail Meat	3	17
	Cooked Grey Mullet (*Mugil cephalus*)	102	18
	Boiled Carp Fillet (*Cyprinus carpio* L.)	1-5	19

2.2 The occurrence and quantitation methods of 2AP in foods

2AP is an important and often a key or characteristic odorant in numerous food products (Table 2.2). It is known to be formed biochemically in aromatic rice,[20] pandan leaves[21] and bread flower.[9] However, its wide occurrence in most foods is attributed to its formation via the Maillard reaction; it is well accepted that 1-pyrroline formed from proline via the Strecker degradation is the key intermediate involved in 2AP formation.[22] Because 2AP can be thermally generated, it exists in a great variety of heated/cooked food products including cooked rice,[4] bread crust,[14] popcorn,[23] sweet corn products,[2] and corn tortillas.[12] In addition to its natural

occurrence in foods, some *Bacillus cereus* strains have been shown to produce 2AP from proline, glutamic acid and glucose.[24]

Table 2.2 Occurrence of 2AP in foods[25]

Biological origin	
aromatic rice	pandan leaves
bread flower	chempedak fruit
Miyabi muskmelon	jackfruit
dry spinach	French beans
Maillard reaction	
bread crust	corn tortilla
roasted sesame seeds	popcorn
cooked lobster meat	cooked beef
cooked mullet	peanut meal
cooked mushroom	boiled potato
Other sources	
Bacillus cereus	
Lactobacillus hilgardii	

Since its initial discovery, researchers have recognized the flavor importance of 2AP and have devised numerous methods for its detection and quantitation. Because of its great instability it is difficult to reliably measure 2AP in foods. To avoid 2AP losses due to degradation throughout the analytical procedure, appropriate extraction and quantification methods should be employed.[25] Continuous steam distillation-solvent extraction (SDE) and purge and trap (Tenax) analysis have been applied as large-scale volatile extraction methods prior to 2AP quantitation by internal standard (I.S.) methodology.[1,4,5,26,27] Bergman and others[6] developed a high throughput method based on dichloromethane extraction of small (0.3 g) ground rice samples followed by GC analysis. Headspace solid phase micro-extraction (HS-SPME) combined with GC analysis is an effective way to screen rice varieties because of the relative high 2AP concentrations in their products.[10,28–30]

Stable isotope dilution assay (SIDA), which compensates for losses during workup and analytical procedures, is the most accurate quantitation method for 2AP determination in foods. Researchers have used both deuterium and ^{13}C labelled analogues of 2AP as I.S. in SIDA. The labelling position and the types of labelled atoms on the 2AP isotope are of great importance. De Kimpe and others[31] demonstrated the synthesis of 2-(acetyl-d_3)-l-pyrroline. However, proton/deuterium exchange has been observed for the 2AP isotope with deuterium labelling on methyl group in protogenic solvent.[29] Schieberle and others were first to develop a SIDA method to quantitate 2AP in wheat and rye bread crust,[14] popcorn[13] and peanut meal[16]. The labelled 2AP was synthesized according to the method of Buttery and coworkers[4] by deuterating 2-acetylpyrrole to form a ring-deuterated 2AP analogue. Elsewhere, Maraval and others[29] developed a synthesis approach starting from L-glutamic acid to produce ring-deuterated 2AP for SIDA combined with HS-SPME and GC-MS quantification. The method enabled the sensitive and accurate quantitation of 2AP in rice plant tissues and grains.

Schieberle and Grosch[14] observed slow proton/deuterium exchanges from ring-deuterated 2AP in a model mixture. For this reason, ^{13}C labelled analogues of 2AP are considered to be the most appropriate internal standards for SIDA of 2AP. Yosihashi and coworkers[20] synthesized ^{13}C labeled 2AP following procedure developed by De Kimpe and others[31] from iodomethane-^{13}C, to facilitate quantitation in aromatic rice stored under different storage conditions. However, just one carbon was labeled making routine GC-EI-MS analysis difficult. Recently, Cadwallader's group first synthesized $^{13}C_2$-2AP with two carbons labelled at the methyl group and carbonyl group, respectively; this new I.S. was applied to quantitate the 2AP recovery from instant rice "flavored" by 2AP zinc chloride complex (2AP-$ZnCl_2$) after the cooking process and provided

great sensitivity in quantitation.[32] To the best of our knowledge, the ^{13}C labelled analogues of 2AP is the most suitable I.S. for 2AP quantitation in foods so far.

Even with SIDA, one must consider the chemical stability of both the unlabelled target compound and isotopically labelled internal standard, as well as the extraction efficiency for tightly bound or matrix entrapped 2AP. Careful method development has allowed researchers to obtain maximum 2AP extraction from rice by several methods. When using an organic extraction method (ethanol or dichloromethane), the highest recovery was found at elevated extraction temperatures (75°C or 80-90°C).[6,20] Temperatures above the gelatinization temperature of rice starch (63.7°C) are required to release 2AP from the starch complex.[20] While harsh conditions are necessary to release 2AP for quantitation, some 2AP may be lost in the process due to degradation. Another quantitation method capable of measuring high levels of 2AP (ppm) in rice seed at room temperature was by extraction into 0.1 N hydrochloric acid.[33] After filtration, the solution pH was made slightly basic and then the 2AP was extracted into dichloromethane.

2.3 2AP stabilization

Buttery mentioned the highly unstable nature (not confined to neat or pure solutions) of 2AP in his early reports,[4] and for this reason researchers have continued to develop ways to improve its stability; however, even today this molecule is scarcely used in commercial flavor formulations. Many attempts have already been made to increase the stability of 2AP including various chemical and physical approaches. Buttery and others[34] patented a chemical process of making stable solid HCl salts of 2AP, whereby the free 2AP molecule could be released from the salt upon contact with materials having basic or buffering properties normally present in many

foods. De Kimpe and Keppens[35] developed a new synthesis method for a stable diethyl acetal of 2AP as its protected analogue, from α,α-diethoxyimine in the presence of lithium diisopropylamide followed by acidic and basic workup, with 92% purity and good yield. Approximately a 1:1 mixture of 2AP and its isomer could be released upon hydrolysis of the protected analogue using a large excess of hydrochloric acid. Morere and others[36] synthesized a stable ketal precursor of 2AP from glutamic acid by a Dakin-West reaction followed by hydride treatment, and 2AP was gradually released from its stable ketal precursor via hydrolysis in weak acid or neutral media with spontaneous oxidation. However, none of above work presented any stability data for 2AP. Duby and coworkers[37–39] adopted both chemical and physical means to improve its stability. 2-(1-Ethoxyethenyl)-1-pyrroline was synthesized and used as a stable precursor of 2AP, then hydrolyzed with acid and neutralized with base, followed by the addition of cyclodextrin solution before freeze drying to obtain powdery microcapsules. However, the 2AP with 10% loading in microcapsules decomposed by 91% after 13 days at 20°C and by 13% after 23 days at 4°C.

Meanwhile, most efforts to stabilize 2AP have involved the use of physical processes. Apintanapong and Noomhorm[40] obtained microcapsules containing 2AP by spray drying using gum acacia and maltodextrins as wall materials. Although the method provided good protection of 2AP; the initial 2AP loading was very low (0.003%). Similarly, Srinivas and others[41] incorporated 2AP in carrier material by vacuum shelf drying or spray drying; however, the 2AP loading was very low (0.05%) and no stability data were provided. Degenhardt and coworkers[42] patented a spray-drying encapsulation (maltodextrin or β-cyclodextrin) process for pandan leaf extracts containing 2AP, but no stability data was provided. Although the above efforts improved the stability of 2AP to some extent, all suffered from at least one of the following deficiencies: 1)

stability of 2AP was either not mentioned or was insufficiently studied; 2) only low loadings of 2AP were achieved; and 3) 2AP was only conditionally stable at low temperature (4°C and -20°C) storage.[43]

Our lab has recently pioneered a novel and attractive stabilization method, whereby 2AP is coordinated as a ligand onto a zinc halide forming a stable powdered complex with excellent 2AP stability when stored in a low moisture environment.[43] Free 2AP can be released upon hydration of the complex. The 2AP zinc iodine complex (2AP-ZnI_2) was stored at three different temperatures (-20°C, 10°C and 25°C) at dry conditions and the stability was measured over a period of 3 months (Figure 2.2). As expected, lower temperature storage conditions favored stability. 2AP content was retained by 97% in the complex after 92 days of -20°C storage, and a 96% retention was observed for 10°C storage. Meanwhile, 2AP content declined by only 6% after 3 months of storage at 25°C.[32,44] Therefore, 2AP-ZnI_2 complex stored at three different temperatures showed excellent stability. This was also the first time 2AP showed almost no degradation when stored under ambient temperature conditions; which suggested the possibility of enabling its use as a flavoring agent. However, an important disadvantage was that the complex degraded immediately in the presence of water, resulting in rapid 2AP release along with obvious color (redness) and texture (clumping) changes (Figure 2.3).

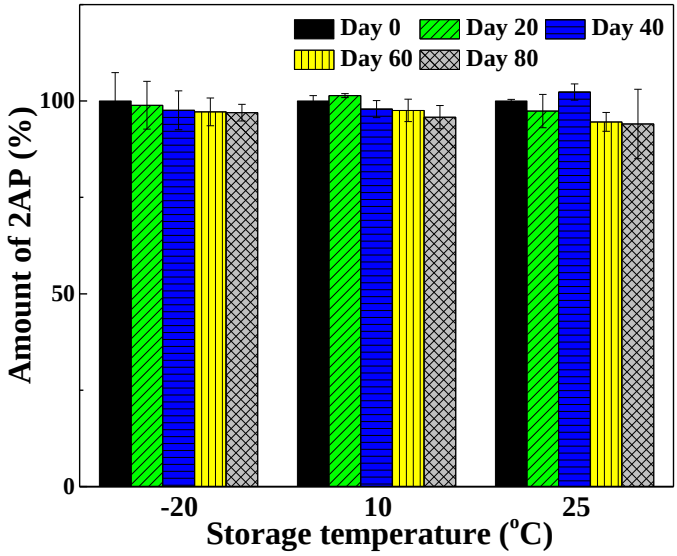

Figure 2.2 Stability of 2AP in 2AP-ZnI_2 complex during storage at -20, 10 and 25°C in desiccators, respectively (mean ± SD, n = 2).[32,44]

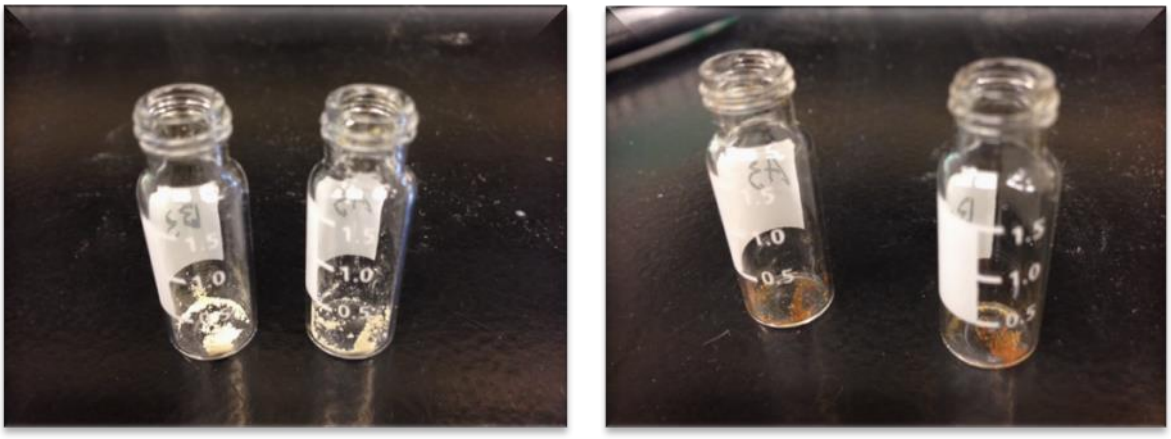

Figure 2.3 2AP-$ZnCl_2$ immediately after complexation (left) and after exposure to ambient condition (38% RH, 21°C) for 15 min.

2.4 Spray-chilling encapsulation

Encapsulation of flavors has been investigated and commercialized using many different methods such as spray-drying, spray-chilling, extrusion, freeze drying, coacervation and molecular inclusion.[45] The choice of appropriate microencapsulation technique depends upon the physiochemical properties of active and wall materials as well as the end use of the product.

Among all above mentioned techniques, spray-chilling is the least expensive encapsulation technology once commercialized and is routinely used for the encapsulation of flavors and functional ingredients to improve heat stability, delay release in wet environments, and convert liquid hydrophilic ingredient into free flowing powders.[46] During the process, the core material is dispersed in a molten coating or wall material and atomized through a high pressure nozzle or spinning disk, and the droplets are solidified once in contact with the cooling gas and liquid and the fine particles are collected as final product.[45,47,48] The initial set-up of spray cooling is quite similar to spray-drying, but no water is evaporated here, which is a significant advantage for encapsulating water sensitive or liable actives. The process is also suitable for protecting many water soluble materials that may be volatilized or damaged during thermal processing. Additionally, spray-chilled microcapsules hold great market potential in bakery products and dry soup mixes.

In the past few years, spray chilling technique was frequently adopted in stabilizing functional ingredients. Tocopherols microcapsules were prepared by spray-chilling using fully hydrogenated soybean oil and soybean oil, high encapsulation efficiency (above 90%) and high storage stability (above 94%) at various conditions were achieved.[49] Similarly, ascorbic acid microparticles were produced using interesterified fat by spray-chilling, the encapsulation

efficiency ranged from 59 to 73%, and the stability of the ascorbic acid after 60 days of storage exceeded 70%; the spray-chilling microencapsulation technique was therefore effective in maintaining the stability of ascorbic acid.[50] Interesterified fat was also applied in lycopene microencapsulation by use of spray chilling, and the incorporated lycopene showed great stability during storage.[51]Additionally, spray-chilling technique was also used to encapsulate vitamin D_3 using vegetable fat as carrier, and the obtained microcapsules loaded with 0.1% active showed improved stability (86.3% vitamin D_3 retention) after 65 days' storage at 25°C, compared to the non-encapsulated vitamin D_3 (60.8%).[52] Besides vitamins, probiotics are commonly seen core actives for microcapsules produced from spray-chilling technique. The cells of *Bifidobacterium animalis* subsp. *lactis* (BI-01) and *Lactobacillus acidophilus* (LAC-04) were encapsulated in cocoa butter, the results suggested that the spray-chilling process protected *L. acidophilus* from gastrointestinal fluids, while the viability of the cells was not affected.[53] Similar results were obtained by Pedroso et al (2012); spray-chilling was considered as an innovative technique to encapsulate *Bifidobacterium lactis* and *Lactobacillus acidophilus* because of the unaffected viability, as well as the protection provided for *L. acidophilus* against simulated gastric fluid and intestinal fluid.[54]

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Chapter 3

EVALUATION OF THE POTENTIAL USE OF THE 2-ACETYL-1-PYRROLINE ZINC CHLORIDE COMPLEX AS A FLAVORING AGENT IN INSTANT RICE

3.1 Abstract

2-Acetyl-1-pyrroline (2AP) is an important contributor to the flavor in many foods. However, 2AP is scarcely used by the flavor industry for use in food formulations due to its great instability. Numerous attempts have been made to increase the stability of 2AP, but until now none has been satisfactory enough to enable the commercial availability or use of 2AP. Our lab pioneered a novel method to stabilize 2AP in powdered form via coordination to a zinc halide. And 2AP-$ZnCl_2$ was chosen for this study because it consists of Generally Recognized as Safe (GRAS) substances. The feasibility of use of this complex to improve the flavor profile for instant rice was investigated in this chapter and the high recovery of 2AP after cooking process (92%) validated its advantages for future potential commercialization.

3.2 Introduction

2AP is an important aroma compound in aromatic rice where it imparts a roasty, popcorn-like aroma. Its main biological sources include aromatic rice (*Oryza sativa*, L)[1], Pandan leaves (*Pandanus*)[2] and bread flower (*Vallaris glabra* Ktze)[3]. 2AP can also be formed via the Maillard reaction and it exists in a great variety of thermally-treated foods including cooked rice[4],

bread crusts[5], popcorn[6] and sweet corn products[7], and corn tortillas[8]. Despite its overall low concentrations in foods, 2AP still plays an important role because of its low odor detection threshold of 0.1 parts-per-billion in water[9]. 2AP is of potential commercial interest to the flavor industry because of its unique and pleasant aroma attributes; however, its great instability has hindered its widespread commercial use.

Many efforts have been made to improve the stability of 2AP, using both chemical[10–12] and physical methods.[12–14] However, none of them was satisfactory enough to provide opportunity for real food applications. Recently, a novel and attractive method was developed in our lab to stabilize 2AP in powder form via coordination to a zinc halide, the free 2AP can be released upon hydration of the complex.[15] The chemical characteristics and storage stability of 2AP-ZnI_2 complex were studied. Surprisingly, the complex was stable after 3 months storage at 25°C, with 2AP degradation only 6%. The complex showed great commercialization potential because of its excellent stability at ambient temperature; however, it degraded immediately in the presence of moisture. Therefore, coating the complex with a water protective hydrophobic layer in order to enable its practical use would be the reasonably next step. However, the feasibility and potential use of the complex as a flavoring agent in real food matrix should be evaluated prior to improving its stability by spray-chilling encapsulation or other means, to ensure the practical significance of the overall objectives of this research project. Meanwhile, among all the 2AP zinc halide complexes studied in our lab, 2AP-$ZnCl_2$ was chosen for food application because both 2AP and $ZnCl_2$ are GRAS substances.

On the other hand, rice is an important food crop, and in many parts of the world aromatic rice varieties are preferred due to their high content of 2AP. For the purpose of

convenience, rice is sometimes sold in an instant form; however, the par boiling process used to produce instant rice removes most of the aroma compounds causing the product to have a bland flavor. In fact, it is common practice to use pandan leaves, a biological source of 2AP, in the cooking of non-aromatic rice varieties to impart stronger aromatic rice-like character to the cooked rice. Therefore, being able to impart aromatic flavor to bland rice by adding 2AP-$ZnCl_2$ complex during the cooking process could greatly improve the sensory properties and consumer acceptance of the rice products and broaden the commercial use of the complex. In this study, a practical application of the 2AP-$ZnCl_2$ complex was demonstrated for the first time for the flavoring of instant rice.

3.3 Materials and Methods

3.3.1 Materials

Pyrrolidine, sodium persulfate, potassium cyanide, tert-butanol, trimethylamine, iodomethane, iodine, magnesium turnings, zinc chloride (1.0 M solution in diethyl ether) were purchased from Sigma-Aldrich (St Louis, MO, USA). Silver nitrate, sodium hydroxide, ammonium chloride, sodium sulfate, hydrochloric acid, anhydrous diethyl ether, pentane and methylene chloride were obtained from Fisher Scientific (Fair Lawn, NJ, USA). 2AP was synthesized from pyrrolidine by Grignard reaction and the purity was above 90% following the procedure described by De Kimpe and Fang.[16,17] The nonvolatile impurities after 2AP synthesis was removed by high vacuum transfer.

2AP-$ZnCl_2$ complex was obtained by adding $ZnCl_2$ solution (1.0 M) dropwise to 2AP etheric solution in a 50 mL centrifuge tube in ice-water bath with good stirring and nitrogen

purging within 1 min, a significant amount of white precipitates formed during addition and the powdery complex was obtained after gentle nitrogen purge to remove the solvent[15]. The complex was stored dry at -20°C in a vial equipped with a PTFE-lined silicon cap. Corn starch was purchased from EM Science (Gibbstown, NJ, USA), and was baked at 130°C for 6 hours prior to use. Uncle Ben's enriched long grain instant white rice was produced by Mars Food US (Rancho Dominguez, CA, USA) and was purchased from a local supermarket.

3.3.2 Methodology

2AP-$ZnCl_2$ complex (10% w/w 2AP loading, 1.5 mg) was dispersed in 5 g of dry corn starch (0.0030% w/w final 2AP loading) to form a free flowing powder (Figure 3.1(a)). The complex-starch dispersion (130 mg) was added to 10 g of instant rice in a 50 mL vial. After addition of 25 mL of water, the vial was sealed with a PTFE-lined cap and then heated by submerging the vial in a boiling water bath for 5 min (Figure 3.1 (b) (c)). Controls consisted 130 mg of the complex-starch dispersion plus phosphate buffer (35 mL, pH 6.2) or 10 g instant rice plus 25 mL water (without complex-starch dispersion)[18]. These were heated using the same procedure described above.

For the analysis of 2AP, vials were first spiked with labelled $^{13}C_2$-2AP (5.12 µg) as internal standard and then analyzed by static headspace solid-phase microextraction (HS-SPME)-GC-MS. Labelled $^{13}C_2$-2AP was synthesized following the procedure described previously[16], except for replacing potassium cyanide and iodomethane with ^{13}C labelled reagents. Vials were pre-incubated for 10 min at 60°C followed by exposure of a 1 cm 50/30 divinylbenzene/carboxen/PDMS fiber (Supelco, Bellefonte, PA, USA) to the headspace for an

additional 20 min (Figure 3.1 (d)). Volatile were desorbed (260°C; splitless, 4 min valve-delay) into a 6890/5973N GC-MS (Agilent Technologies, Inc., Palo Alto, CA, USA). Separations were performed using a Stabilwax column (30 m x 0.25 mm i.d. x 0.25 µm film) with helium (1 mL/min) as carrier gas. GC oven was programmed from 40°C (5 min initial hold time) to 225°C (40 min final hold time). MS was operated in the EI-SIM mode. Quantitation was done by comparing the ratio of the selected ion peak areas for unlabelled 2AP (ion 111) and $^{13}C_2$-2AP (ion 113) by use of a response factor of 1.03.

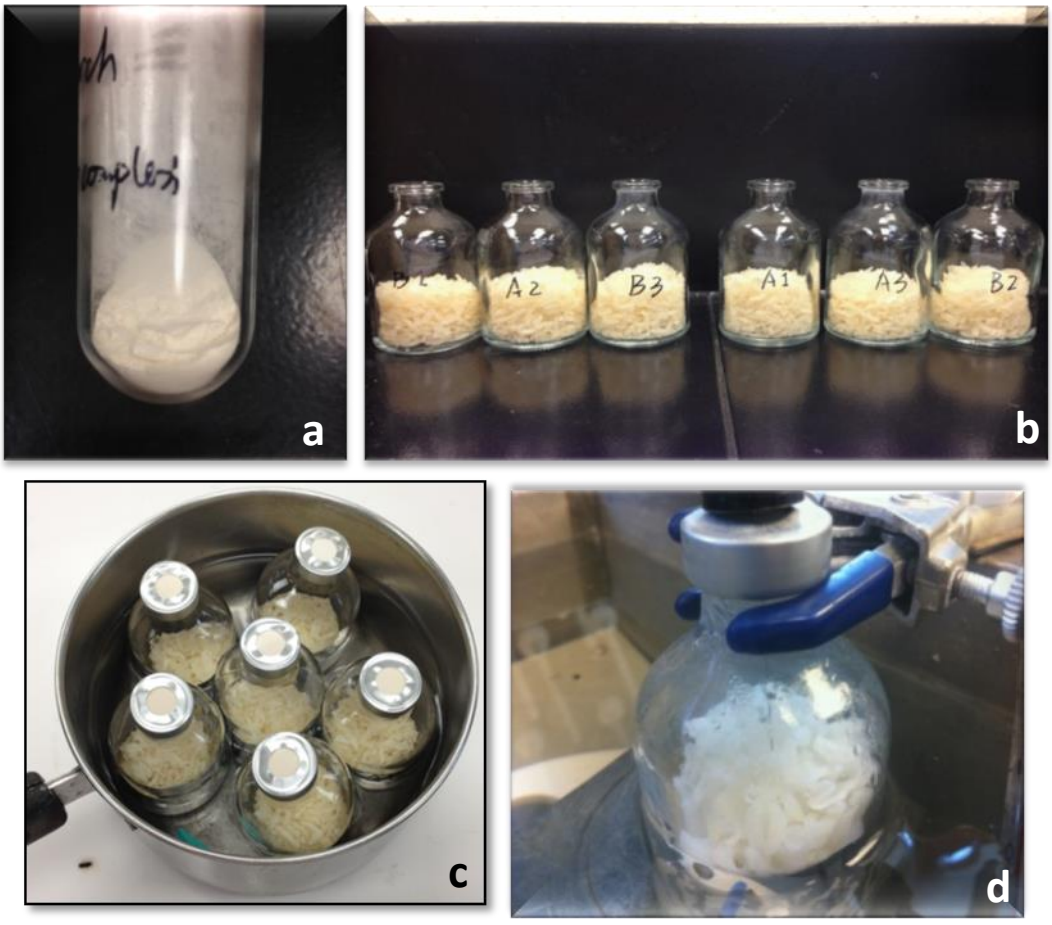

Figure 3.1 (a) 2AP-$ZnCl_2$ complex was dispersed in dry corn starch, (b) instant rice samples were in 50 mL vials, (c) instant rice samples were heated after sealing with PTFE-lined caps in a boiling water bath, (d) HS-SPME extraction of aroma from cooked rice samples.

3.4 Results and Discussion

This study was conducted to determine the feasibility of using the 2AP-$ZnCl_2$ complex to add aromatic rice flavor to bland instant rice during the cooking process. The results are shown in Table 3.1. For cooked instant rice without added complex the final 2AP concentration was found to be only 3.97 ng/g, confirmed this instant rice to be non-fragrance rice. On the other hand, the instant rice prepared with the complex contained 105 ng/g of 2AP. This was within the range expected for commercial aromatic rice varieties (19-999 ng/g).[19] In addition, a 92% recovery of 2AP was obtained for the use of the complex in the instant rice, which indicated only a slight loss of 2AP occurred during cooking process. Therefore, the feasibility of applying 2AP-$ZnCl_2$ complex as a flavoring agent to commercial food products like instant rice was clearly demonstrated.

Table 3.1 2AP concentrations in cooked instant rice "flavored" with 2AP-$ZnCl_2$ complex dispersed in dry starch (mean, n = 2).

Treatment	2AP found (µg)	Concentration of 2AP (ng/g)	2AP Recovery (%)
Buffer + complex	3.85	110	100
Cooked rice (no complex)	0.141	3.97	NA
Cooked rice + complex	3.67	105	92

Very few studied attempted to apply 2AP as a flavoring agent in food matrices. An aqueous solution containing 0.05 ppm synthetic 2AP was added to unscented cooked rice and the addition provided the rice having the aroma of the "scented" rice variety.[20] This study was the first one incorporated 2AP in food application, although sensory evaluation was performed, the flavor recovery of 2AP was not determined. In another patent, a coating composition

manufactured from scented rice containing at least 40 ppb 2AP was used in flavoring food products through the cooking or frying process.[21] It claimed that an improved sensory profile was achieved after including the coating composition containing 2AP molecule; however, neither flavor recovery data nor sensory evaluation results was quantitatively available to prove the effective application of that flavoring agent. Therefore, up to now, this study was the first one quantitated the flavoring effectiveness of 2AP in real food scenarios, and the promising potential of this complex being a powerful flavoring agent was exhibited the first time.

3.5 Conclusion

As expected, the release and high recovery of 2AP molecule from this GRAS grade complex during a simulated cooking process was achieved under airtight sealing condition, and the potential of using the complex in real food matrices was confirmed by high flavor recovery. However, the application of unprotected/pure 2AP-$ZnCl_2$ in foods is nearly impossible because the complex readily degrades as soon as it is exposed to ambient atmosphere with water vapor, which prevents the effective handling, storage and application in real foods. This study validated the necessity of encapsulating the highly unstable complex with a hydrophobic layer in order to promote its practical applications.

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Chapter 4

FEASIBILITY OF ENCAPSULATING 2-ACETYL-1-PYRROLINE ZINC CHLORIDE COMPLEX IN WAX BY SPRAY-CHILLING

4.1 Abstract

2-Acetyl-1-pyrroline (2AP) is an important and characteristic odorant in a multitude of foods[1] and is of great commercial interests. Our lab has successfully stabilized this highly unstable molecule by complexation with zinc chloride ($ZnCl_2$) to form a powder using Generally Recognized as Safe (GRAS) substances. The complex was shown to have the potential of being an excellent flavoring agent[2] in the previous chapter; however, it is prone to degrade in presence of moisture, which hinders its widespread application. In this study, the 2AP-$ZnCl_2$ complex was successfully coated by a hydrophobic layer by spray-chilling encapsulation in order to improve its stability during storage and application. At the same time, the spray-chilling apparatus was modified to better meet our purpose; appropriate wax materials were carefully selected and targeted aroma complexes were identified to facilitate the wax complex encapsulation. Free flowing microcapsules with high flavor retention (65.26%) of 2AP were achieved using octacosane as carrier matrix.

4.2 Introduction

2AP is an important odorant found in many foods where it exhibits a pleasant note.[3–10] Although the highly unstable nature of the molecule has prevented its use in flavor industry for

more than 30 years, our lab has developed a method to stabilize it by coordination with $ZnCl_2$.[11] The 2AP-$ZnCl_2$ complex is a powder form and possesses great stability in absence of water, even when stored at ambient temperature. However, it is readily degraded in the presence of moisture and its stability is also negatively influenced by high temperature. Coating the complex with a hydrophobic layer with low water vapor permeability by an appropriate encapsulation technique with mild process conditions was presumed to be able to help retain the 2AP stability.

Due to the nature of our 2AP-$ZnCl_2$ complex, encapsulation techniques such as fluidized bed coating, spray-chilling and melt injection are considered since they could produce microcapsules in the absence of water involved throughout the whole process. Spray-chilling was chosen in this study since it is the least expensive encapsulation process once commercialized and is routinely used for encapsulation of aroma compounds to improve heat stability, delay release in wet environments, and convert liquid flavor into free-flowing powders.[12] During the process, a molten wall material is mixed with the core active and then atomized, cooled and solidified to form the final powder.[13–15] An important consideration in spray-chilling is the selection of suitable encapsulation wall materials that can undergo solidification in response to the relatively low temperatures of cold air. It is necessary to maintain a chilling chamber temperature sufficiently below the melting temperature of the wall materials.

Wax materials with low water vapor permeabilities are required in this study in order to prevent the labile complex from moisture attack. According to Donhowe and Fennema (1993), among many of edible films, the permeabilities of candelilla wax and paraffin wax at 25°C are 0.18 and 0.22 $g[m \cdot s \cdot Pa]^{-1} \times 10^{-12}$, respectively, lower than that of carnauba wax, beeswax,

microcrystalline wax, acetylated monoglycerides, chocolate, shellac, acetylated monoglycerides.[16] Besides that, other physical properties such as melting point, food grade availabilities and powder form should also be considered.

Several complexes such as 2-acetylpyridine zinc chloride (2APri-$ZnCl_2$), 2-acetyl-2-thiazoline zinc chloride (2A2T-$ZnCl_2$) and 2AP-$ZnCl_2$ were tested in spray-chilling trials, in order to facilitate the ultimate successful production of 2AP-$ZnCl_2$ microcapsules. The stable complex 2APri-$ZnCl_2$ was used to investigate the performance of spray-chilling system, while the unstable complex 2A2T- $ZnCl_2$ was applied as a model to avoid possible degradation caused during the process.

4.3 Materials and Methods

4.3.1 Materials

2-Acetylpyridine (2APri), 2-acetyl-2-thiazoline (2A2T), 2,4,6-Collidine, zinc chloride (1.0 M solution in diethyl ether), bromoform and C_{28} paraffin (Octacosane) were purchased from Sigma-Aldrich (St Louis, MO, USA). 2-Acetylthiazole was provided by Bedoukian Research Inc (Danbury, CT, USA). Sodium sulfate, anhydrous diethyl ether, pentane and methylene chloride were obtained from Fisher Scientific (Fair Lawn, NJ, USA). 2AP was synthesized following the same protocol described in Chapter 3. The nonvolatile impurities from 2APri, 2A2T and 2AP etheric solutions were removed by high vacuum transfer prior to coordination with $ZnCl_2$. Candelilla wax, carnauba wax, beeswax and microcrystalline wax used in this study were kindly provided by Koster Keunen, Inc (Watertown, CT, USA). Paraffin waxes SP-206, SP-192P and SP-173P were kindly supplied by Strahl & Pitsch, Inc. (West Babylon, NY, USA). Corn starch

was purchased from EM Science (Gibbstown, NJ, USA), and was baked at 130°C for 6 h prior to use.

4.3.2 Spray-chilling process

A laboratory scale Mini Spray-dryer B-290 (Büchi Corporation, Switzerland) was used [Figure 4.1 (a)] to encapsulate the flavor complexes. The spray dryer was equipped with a spray-chilling accessory, 230V (040351), a dehumidifier (B-296) and a two-fluid nozzle (nozzle cap, Ø=2.2mm; nozzle tip, Ø=1.4mm; 046376). The spray-chiller was operated according to the manufacturer's recommendations with atomization airflow set to 30 mm rotameter (439 L/h) using compressed nitrogen at 80 lb/in^2 pressure.

The general spray-chilling process started with preheating of wax using a heating mantle [Figure 4.1 (b)]. A thermometer was used to indicate the target temperature of the melted wax before the complex was added, after which the mixture was stirred using POLYTRON® PT 10 homogenizer (Kinematica AG, Switzerland) [Figure 4.1 (c)] at 20000 RPM for 10 seconds before feeding to the sample holder of the spray-chilling accessory. The temperature of the wax and complex mixture was kept in the sample holder (via the heating bath of spray-chilling accessory) at 40% above the wax melting point according to the manufacturer's recommendation. The mixture was then atomized by the nozzle to form small droplets which solidified once in contact with the cooling air in the chamber to yield a powdered product, which was finally collected after the cyclone in the sample vessel [Figure 4.1 (d)].

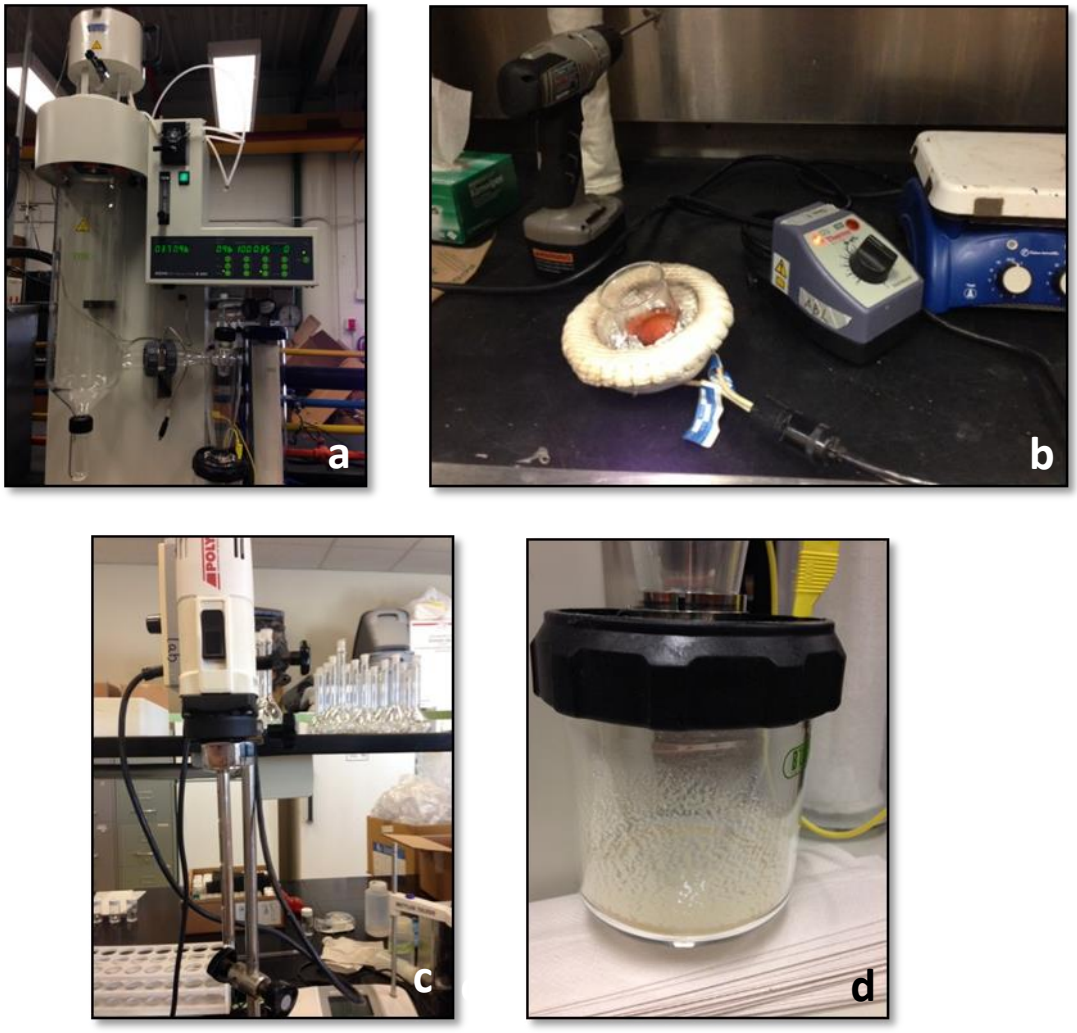

Figure 4.1 (a). Mini Spray-dryer B-290 (Büchi Corporation, Switzerland) used in this study; (b). The wax was preheated by a heating mantle before mixing with the complex; (c). The POLYTRON® PT 10 homogenizer (Kinematica AG, Switzerland) used for mixing the melted wax and complex; (d) Powdery microcapsules were collected from sample vessel.

4.3.3 Modification of the initial design of the spray-chilling accessory

The initial design of the spray-chilling accessory with a flat bottom sample holder was not appropriate for our purpose. The difference in densities between waxes and complexes were significant: the densities of the melted waxes ranged from 0.81 g/mL to 0.95 g/mL, according to

the wax specification sheets provide by the manufacturers; while the density of the complexes were between 1.33 g/mL and 2.89 g/mL, from lab experimental data. A large portion of the complex was retained at the bottom of the holder without being transferred to form microcapsules, even when homogenization was appropriately applied, due to the large density differences between the complex and wax, as well as the flat bottom of the sample holder; which greatly affected the flavor retention of the process and reduced the flavor loading of microcapsules.

A new design of the sample holder was required to replace the cylindrical one in order to fully transfer the complex to be encapsulated with wax. Figure 4.2 showed the design schematics for the new funnel adapted to the original spray-chilling accessory.

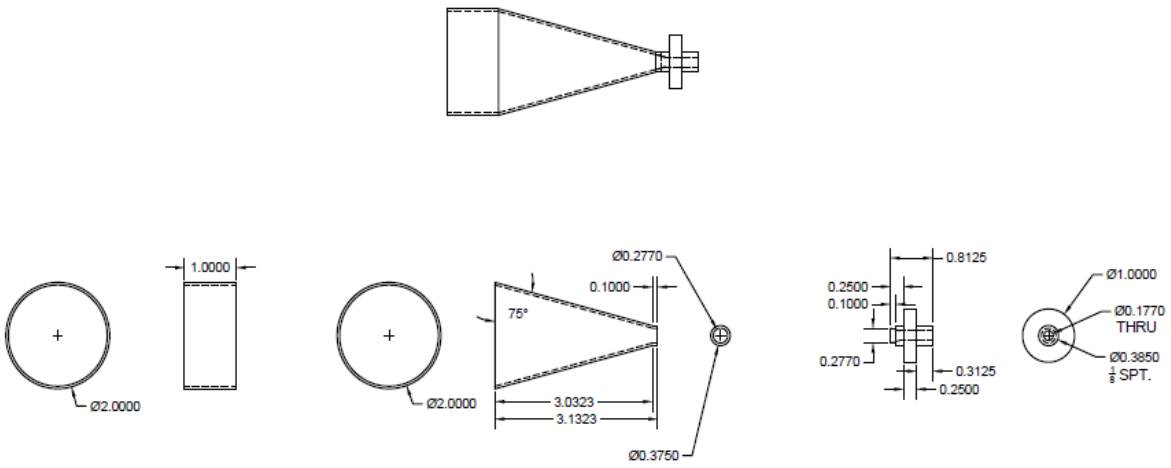

Figure 4.2 Design schematics for the new funnel adapted to the original spray-chilling accessory (adapted from ECE machine shop at University of Illinois at Urbana-Champaign).

4.3.4 Wax selection for spray-chilling encapsulation

Our targeted 2AP-$ZnCl_2$ complex is highly unstable with moisture presence[11]; therefore, it is critical to choose a wax with low water vapor permeability to be the wall material for the microcapsules, in order to serve as effective moisture barriers and protect the core complex from degradation. Meanwhile, waxes having low melting points are preferred, not only because high temperatures negatively affect the stability of the complex[11], but also because melting points for some waxes may exceed the upper temperature limit of the spray-chilling accessory. Most importantly, the wall materials should be made of food grade materials since the final application of the flavoring agent will be in food matrices. Finally, a free flowing nature of the final products from spray-chilling is preferred to ensure the shelf life and facilitate ease in applications. The free flowing properties of waxes after spray chilling were examined not only by observation of human eyes but also with the aid of scanning electron microscopy (SEM). An environmental scanning electron microscope (ESEM) with a field-emission electron gun (XL30 ESEM-FEG; Philips/FEI Co., Hillsboro OR) was used to evaluate the surface morphology of the microcapsules. It was operated in high-vacuum SEM mode at 5 KV, spot 3 (2.1 nm). The above criteria were considered when choosing the appropriate waxes. Candelilla wax, carnauba wax, beeswax and microcrystalline wax, paraffin waxes SP-206, SP-192P and SP-173P were obtained and their physical properties were carefully evaluated based on the criteria.

4.3.5 Model complexes selection for encapsulation

Besides 2AP, some other heterocyclic compounds such as 2APri and 2A2T could be coordinated as ligands onto zinc halide and form powdered complexes[11]. Among all these

complexes, 2APri-$ZnCl_2$ exhibits good chemical and physical stability under ambient conditions; therefore, it was chosen as a suitable model complex for investigating the performance and working parameters of the spray-chilling apparatus. Obviously, the spray-chilling technique would not be a feasible approach if the stable 2APri-$ZnCl_2$ could not be successfully encapsulated. A second complex, 2A2T-$ZnCl_2$ also possesses great instability (much like 2AP-$ZnCl_2$) and is prone to degradation under ambient conditions. However, 2A2T is commercially available and for this reason less effort is needed to prepare the free-flowing complex. It was therefore studied as an unstable complex model to test and solve the degradation issues identified in the process and to investigate the overall effectiveness of wax complex encapsulation. Our ultimate complex 2AP-$ZnCl_2$ could be obtained by a series of time-consuming synthetic and complexation steps; proper sample preparation and spray chilling conditions without degrading the liable complex are essential to assure the successful production of 2AP-$ZnCl_2$ spray-chilled microcapsules.

4.3.6 Determinations of flavor loading in microcapsules and flavor retention from spray-chilling encapsulation process

Flavor loading of each type of microcapsules was determined using multiple extraction steps combined with gas chromatography (GC) quantitation using internal standard (I.S.) method. Fifty milligram of microcapsules coated with candelilla wax were heated at 85°C in a test tube for 3 min, 5 mL phosphate buffer (50 mM, pH 7) at the same temperature were added and vortexed for 1 min to release the aroma from the complex. The mixture was then cooled down and 5 mL of pentane containing I.S. 2, 4, 6-Collidine at a concentration of 1.08 µg/µl was added

and the test tube was vortexed for another 3 min following by centrifugation at 3000 rpm for 5 min. The upper solvent layer was collected and dehydrated by sodium sulfate before GC injection. Since paraffin waxes have much higher solubilities in anhydrous diethyl ether compared to candelilla wax, the protocol of flavor loading determination for microcapsules made from paraffin waxes were less complicated. Figure 4.3 shows the general protocol for flavor loading determination for microcapsules coated with paraffin waxes, ether was used to melt the wall materials of the microcapsules to facilitate the analysis. Flavor retention from the spray chilling process was measured based on the flavor loading of microcapsules and the theoretical flavor content in wax and complex mixture, its calculation was done using equation 1.

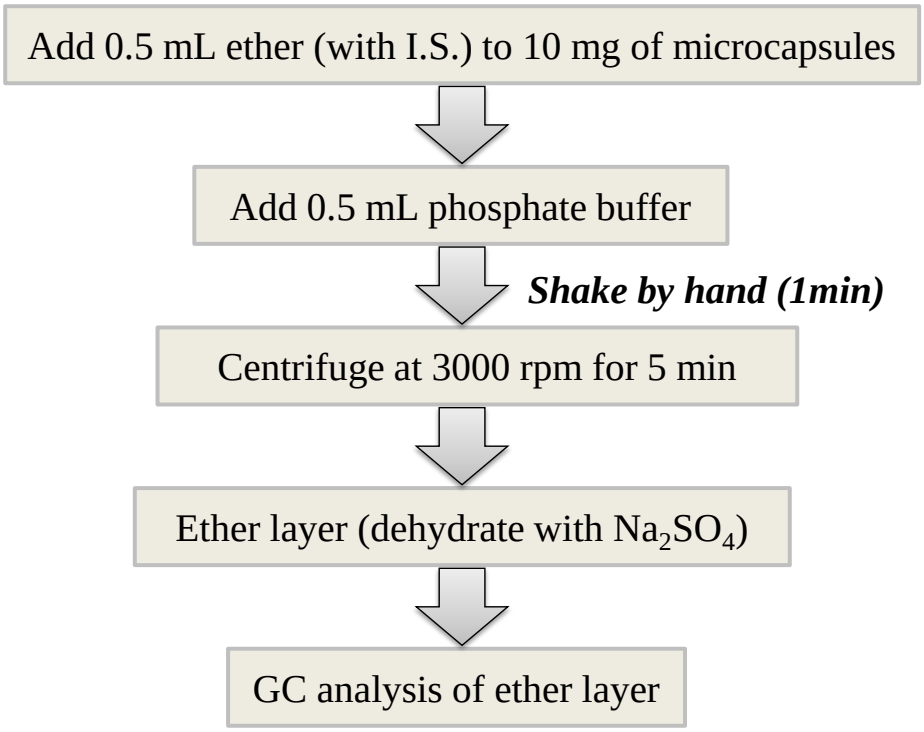

Figure 4.3 General protocol of flavor loading determination for spray-chilled microcapsules walled with paraffin waxes.

$$\text{Flavor retention (\%)} = \frac{Flavor\ \ loading\ \ in\ microcapsules}{Theoretical\ \ flavor\ \ content\ \ in\ feed\ \ mixture} \times 100\% \quad (1)$$

4.3.7 Gas chromatography

Flavor analysis for 2APri-$ZnCl_2$ spray-chilled microcapsules was performed using a 5890 series II GC equipped with a split/splitless injector, flame ionization detector (FID), and HP-1 column (30m × 0.32 mm × 0.25 μm film thickness, Agilent); while the measurements for 2A2T-$ZnCl_2$ and 2AP-$ZnCl_2$ microcapsules were performed using a 6890N GC/5973N mass selective detector (MSD; Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a fused-silica capillary column (Stabilwax, 30 m × 0.25 mm × 0.25 μm film thickness; or Rtx-5, 15 m × 0.32 mm × 0.5 μm film thickness; Restek, Bellefonte, PA, USA). Helium was the carrier gas at a constant flow of 1 mL/min. The injector was held at 250°C in the splitless mode. GC oven temperature was programmed from 40 to 225 °C at 10 °C/min with initial and final holding times of 5 and 40 min, respectively. Other conditions for the MSD were as follows: MSD interface temperature, 260°C; ionization energy, 70 eV; mass range, 35-350 amu; EM voltage, Autotune +165 V; scan rate, 4.45 scans/s. Quantitation was done using area ratio, mass ratio and response factor according to equation 2.

$$\text{2AP content (mg)} = \frac{Peak\ \ area\ \ of\ \ unlabelled\ \ \ 2AP\ (111)}{Peak\ \ area\ \ of\ \text{labelled}\ \ 2AP\ (113)} \times concentration\ of\ I.S.\ (mg/mL)\ \times\ 0.5\ mL \times R_f \quad (2)$$

4.4 Results and Discussion

4.4.1 Modification of design of spray chilling accessory

The replacement of the flat-bottomed sample holder with funnel to the spray-chilling accessory (Figure 4.4) facilitated the near complete transfer of the complex to the nozzle. Also,

due to the great instability of 2AP-zinc halide complexes, a more stable complex 2APri-$ZnCl_2$ was used here to demonstrate the effectiveness of using the funnel for feeding the mixture. The flavor retention of 2APri in candelilla microcapsules increased from 47.64 % to 62.16% after replacing the initial sample holder with the new funnel. This improved design showed good performance in terms of effectively feeding the premix to the instrument and the final uniformity of collected microcapsules. Therefore, after modification, the spray-chilling system was presumed to be an appropriate lab scale technique to encapsulate the complexes by wax wall materials with reasonable flavor retention. The equipment parameters were further explored to reach the best performance without degrading the labile complexes during the process. The temperature of the thermostat/water bath for the reservoir was set to approximately 40% above the melting point of each wax. Meanwhile, the homogenization temperature of melted wax and corresponding complex was set at about 6-12°C above the melting point of the wax in order to minimize the thermal degradation of the complex during the sample preparation steps.

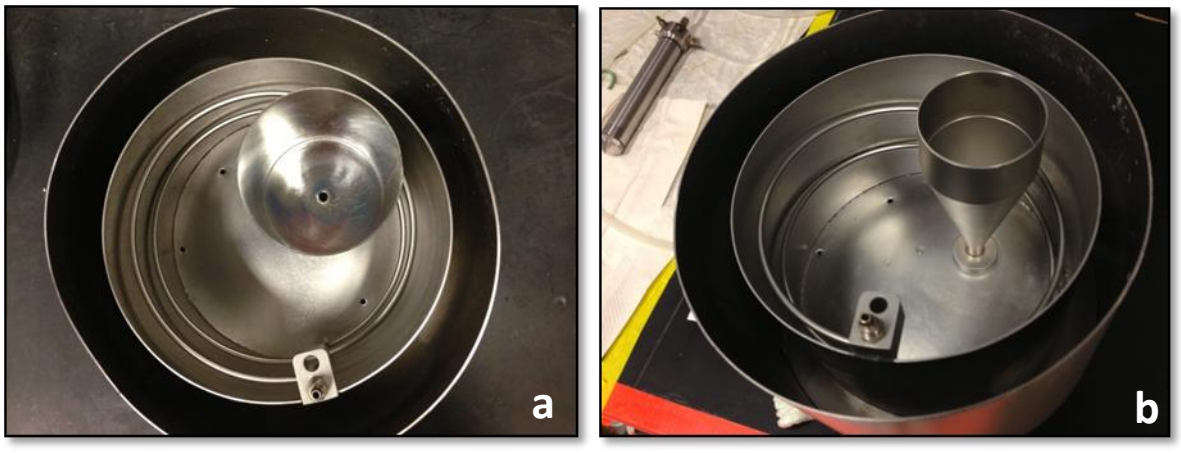

Figure 4.4 Initial cylindrical sample holder (a) and improved funnel sample holder (b) for spray-chilling accessory**.**

4.4.2 Wax selection for spray-chilling process

In order to ensure an effective layer of moisture barrier as well as the free-flowing properties of the final products during storage and use, the properties of potential wax materials were investigated and the selection was carefully decided upon following criteria relevant to realistic scenarios. Table 2 summarizes the physical properties of various waxes evaluated in this study. Carnauba wax was not appropriate because of its high melting point which exceeded the limit of the heating bath of the sample holder of the spray-chilling. The sticky nature of Beeswax made it unsuitable for spray-chilling since it clogged the nozzle without forming fine particles. The high water vapor permeability of microcrystalline wax precluded its use as a moisture barrier. Among all the waxes considered, candelilla and paraffin waxes (including octacosane) exhibit the lowest water vapor permeabilities and also have relatively low melting points. Furthermore, candelilla and octacosane (C_{28} paraffin) both possess brittle natures and formed excellent free flowing powders after the spray-chilling [Figure 4.5 (a) and (b)]. Besides the powdery properties, their surface morphology were observed from SEM graphs [Figure 4.5 (c) and (d)], both octacosane and candelilla formed microspheres with wide particle size distribution ranges. However, it seemed that the candelilla wax showed smoother surface and a narrowed particle size distribution compared to octacosane; therefore, from this viewpoint, candelilla wax was preferred within those two options. Commercial food-grade paraffin waxes (with melting point 50-63°C) also showed free flowing and powdery characteristics immediately after spray-chilling; however, clumps were observed after a week of ambient storage (see Figure 4.6 as an example). Therefore, regular food grade paraffin waxes were not considered as ideal for our purpose. However, they could be a good option if the caking issue is resolved by possibly adding a food grade desiccant or anticaking agent. In conclusion, candelilla wax, general food grade

paraffin and octacosane (C_{28} paraffin) were chosen on the basis on their advantageous properties. Results above helped determine the next steps of wax complex encapsulation without the need for exhaustive exploration.

Table 4.1 Water vapor permeabilities and related properties of wax materials.

Waxes	**Melting point (°C)[a]**	**Permeability at 25°C ($g[m \cdot s \cdot Pa]^{-1} \times 10^{-12}$)[b]**	**Physical properties after spray-chilling[c]**	**Food grade**
Candelilla wax	68-73	0.18	Free flowing powder (Figure 6b)	Yes
Carnauba wax	80-86	0.33	Not appropriate for spray-chilling due to its high melting point	Yes
Beeswax	61-65	0.34	Not appropriate for spray-chilling due to its stickiness property	Yes
Microcrystalline wax	61-92	0.58	Not appropriate for spray-chilling due to its wide range of melting point	Yes
Paraffin wax	51-73	0.22	Free flowing powder, forms clumps after weeks (Figure 7)	Yes
Octacosane (C_{28} Paraffin wax)	57-62	0.22	Free flowing powder (Figure 6a)	Yes

[a]Data obtained from Bennett, 1975.

[b]Data obtained from Donhowe and Fennema, 1993.

[c]Information obtained from lab-scale spray-chilling trials.

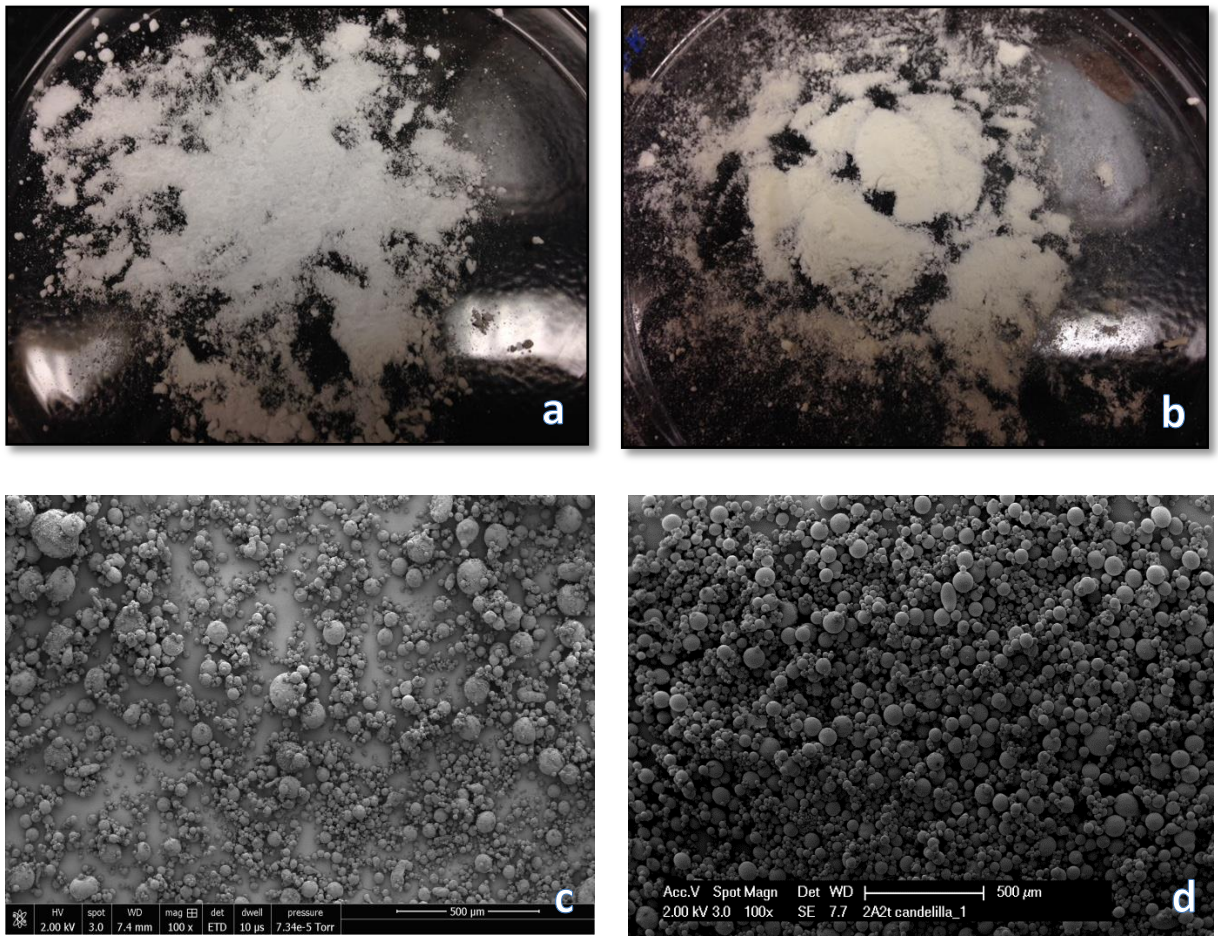

Figure 4.5 Free flowing powders obtained from spray-chilling of octacosane by (a) eye observation; and (c) SEM, candelilla wax by (b) eye observation and (d) SEM**.**

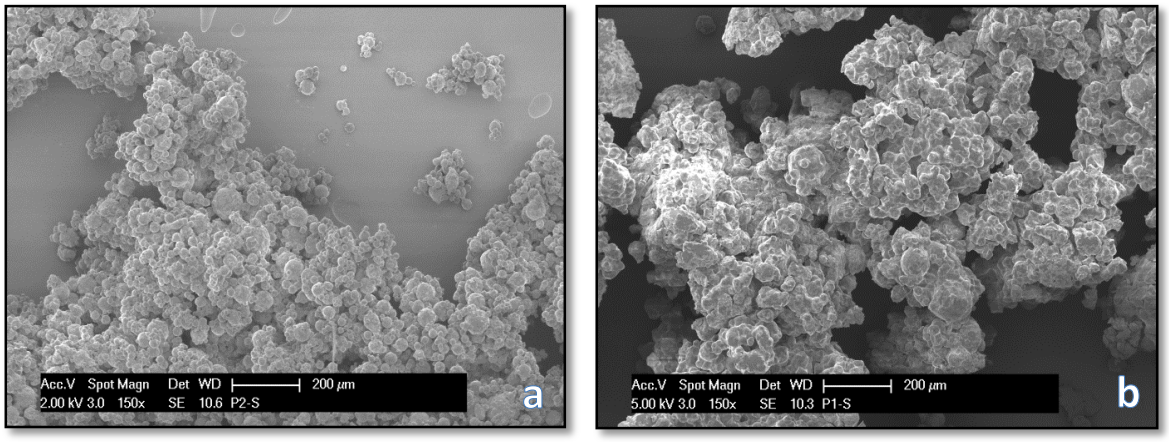

Figure 4.6 SEM graphs of spray-chilled food grade paraffin wax SP-192P (melting point 53°C) (a) on day 0 and (b) day 7 during ambient storage.

4.4.3 Spray-chilling encapsulation of complexes and waxes

The spray-chilling encapsulation trials for selected complexes using various waxes are shown in Table 4.2. As mentioned previously, 2APri-$ZnCl_2$ was used for investigating the performance of the spray-chilling system since it exhibits good chemical and physical stability. The modification of the sample holder resulted in the formation of powdery 2APri-$ZnCl_2$ candelilla microcapsules after spray-chilling, with a flavor retention increased to 62.2% (example 1 in Table 4.2). The unstable model complex 2A2T-$ZnCl_2$ was then used in place of the stable 2APri-$ZnCl_2$ for candelilla wax encapsulation (example 2 in Table 4.2). Unfortunately, even using the same operational conditions and with the unstable complex carefully protected, the flavor retention was only 5.42%. Since the stability of 2A2T-$ZnCl_2$ and 2AP-$ZnCl_2$ is negatively influenced by moisture and temperature, the high mixing temperature required for this wax during sample preparation step might have caused the degradation of the complex prior to spray-chilling. Use of waxes with lower melting points might be a possible solution to this

problem. Paraffin wax SP-206 was chosen because of its low melting point (51°C) which requires relatively low mixing temperature (60-63°C). Example 3 in Table 4.2 shows an increase in flavor retention of 13.21% was achieved by spray-chilling of 2A2T-$ZnCl_2$ with Paraffin SP-206. However, this result was not satisfactory since a higher flavor retention than this would be necessary for practical application. Therefore, other alternatives were required to minimize the thermal degradation of the complex. For this purpose, 2A2T-$ZnCl_2$ was well dispersed in oven-dried corn starch prior to homogenization with melted paraffin SP-206 (example 4 in Table 4.2). The dry starch was expected to serve as a protection layer or moisture desiccant to minimize the thermal degradation of the complex, and it was observed that flavor retention of the microcapsules was greatly increased (32.63%). However, the spray-chilled particles formed clumps during ambient storage, which was accompanied by the degradation of the complex since the microcapsules lost their protective structure of the core substance. The soft and sticky physical properties of paraffin SP-206 might have been the reason for the clumping; therefore, brittle waxes were considered in order to help maintain the stability of the microcapsules. Paraffin SP-192P was observed to have a more brittle property compared to SP-206; consequently, this wax was mixed with 2A2T-$ZnCl_2$ at a slightly elevated temperature (65°C) before spray-chilling, and a flavor retention at 29.02% was achieved (example 5). Similarly, these microcapsules caked in a matter of days under ambient storage conditions and the physical stability issue of final microcapsules still existed. The above results illustrated the need for waxes with even more brittle properties. Therefore, waxes with melting points between 53 and 72°C were evaluated in subsequent studies. Microcapsules produced from paraffin SP-173P (with a melting point of 62°C) resulted in 19.6% flavor retention; however, the microcapsules experienced a loss of flowability during storage, thus eliminating this wax from further

consideration (Example 6). After a series of exploration, octacosane (C_{28} paraffin) was found to be a very brittle wax and has an acceptable melting point range between 57-62°C and was evaluated for its potential being the appropriate wall material for 2A2T-$ZnCl_2$ microcapsules. Example 7 demonstrates an acceptable flavor retention (56.98%) in microcapsules was produced from octacosane and 2A2T-$ZnCl_2$. Furthermore, these microparticles could maintain a free flowing nature under ambient storage for months or even years (lab unpublished data). Therefore, octacosane was demonstrated to be an ideal wax wall material for encapsulating the unstable complexes. As a result, 2AP-$ZnCl_2$ was encapsulated by octacosane to form free flowing power with 65.26% flavor retention (Example 8).

Table 4.2 Spray-chilling encapsulation of targeted complexes by various types of waxes.

Examples	Waxes	Melting point of wax (°C)[a]	Complex	Mixing temperature for wax and complex (°C)	Flavor retention (Mean±SD, %)	Physical properties of microcapsules
1	Candelilla	72	2APri-$ZnCl_2$	80-83	62.2±1.3	Free flowing powder
2	Candelilla	72	2A2T-$ZnCl_2$	80-83	5.42±0.88	Free flowing powder
3	Paraffin SP-206	51	2A2T-$ZnCl_2$	60-63	13.21±0.42	Free flowing powder
4	Paraffin SP-206	51	2A2T-$ZnCl_2$ dispersed in dry starch	60-63	32.6±1.8	Particles formed clumps during storage
5	Paraffin SP-192P	53	2A2T-$ZnCl_2$ dispersed in dry starch	65	29.02±0.81	Particles formed clumps during storage
6	Paraffin SP-173P	62	2A2T-$ZnCl_2$ dispersed in dry starch	68-70	19.58±0.16	Particles lost flowability during storage
7	C_{28} paraffin (Octacosane)	57-62	2A2T-$ZnCl_2$	68	56.98±0.21	Free flowing powder
8	C_{28} paraffin (Octacosane)	57-62	2AP-$ZnCl_2$	68	65.3±3.2	Free flowing powder

[a]Data obtained from specification sheets of sample manufactures.

As we notice, spray-chilling became a frequently-used technique in recent years to encapsulate functional ingredients, convert liquid active into free flowing powders and improve their heat stability. Table 4.3 summarized the recent studies applied spray-chilling techniques.[17–22] Interestingly, all of the above mentioned studies used triglycerides as wall materials for spray-chilling processes, including soybean oil[19], interesterified fat[17,18,22], vegetable fat[20] and cocoa butter[21]. Triglycerides could easily undergo polymorphism. Polymorphism is the ability of the triglyceride molecules possess various crystal forms having significantly different physical properties, and the conversion of one polymorph to another; it is one of the most important physical degradative routes affects the stability of solid forms.[23] For microcapsules produced from triglycerides, the manufacture process such as spraying may specifically trigger polymorphism issue which negatively influences the quality and texture of the end products during shelf storage.[23] To the best of our knowledge, this study was the first one used paraffin waxes as wall materials for spray-chilled microcapsules, which greatly reduced the physical degradation issues caused by polymorphism and better structural-stability was expected. Furthermore, previous studies focus on stabilizing functional ingredients by use of a triglyceride layer; this study firstly demonstrated the feasibility of encapsulating flavor powdery substances by spray-chilling process, which provided a successful example of using this technique in flavor industry.

Table 4.3 Studies applied spray-chilling encapsulation technique in recent years.

Core materials	Wall materials	References
Tocopherols	Soybean oil	19
Ascorbic acid	Interesterified fat	17
Lycopene	Hydrogenated & interesterified fat	22
Vitamin D_3	Vegetable fat	20
Bifidobacterium animalis subsp. Lactis & *Lactobacillus acidophilus*	Cocoa butter	21
Bifidobacterium lactis & *Lactobacillus acidophilus*	Interesterified fat	18

4.5 Conclusion

In conclusion, the feasibility of encapsulating the readily degradable 2AP-$ZnCl_2$ complex by use of wax (octacosane) as wall material was clearly illustrated, which supported the core rationale of this research project. Comparing to pure/unprotected 2AP-$ZnCl_2$ complex, the microcapsules form was easier to handle, transfer and apply in real food matrices, which greatly promoted its potential of being an excellent flavoring agent. However, the chances of discovering waxes with better performance in terms of higher flavor retention and lower manufacture cost still exist and further efforts may be paid to explore other possibilities.

4.6 References

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Chapter 5

CHEMICAL AND PHYSICAL CHARACTERIZATION OF 2-ACETYL-1-PYRROLINE ZINC CHLORIDE MICROCAPSULES

5.1 Abstract

Physical and chemical methods were applied to characterize 2AP-$ZnCl_2$ microcapsules in order to understand their properties and help facilitate future modification. Scanning electron microscopy (SEM) and X-ray micro-computed tomography (CT) were applied to investigate the morphological properties of the microcapsules. Meanwhile, appropriate methods for determining 2AP and $ZnCl_2$ contents in microcapsules were developed by use of gas chromatography (GC) and absorbance spectroscopy measurement, respectively. The results demonstrated that the microcapsules possessed desirable characteristics for potential food application.

5.2 Introduction

The knowledge and understanding of the physical and structural properties of microencapsulated flavors are essential to reduce cost and to obtain an optimum process and product functionality (stability, release, and sensorial perception). Therefore, reliable analytical techniques should be used to characterize flavor encapsulates.

Physical characterization provides a detailed understanding of the surface morphology and structural properties of flavor microcapsules. These characteristics may be impacted by the method of manufacture and processes variables. A wide array of analytical techniques are available and utilized for the physical characterization encapsulates, the most common methods being microscopy techniques including scanning electron microscopy (SEM). SEM is a testing tool widely applied for investigating the physical structure especially the morphology of flavor microcapsules.[1–5] It permits imaging of structures with a greater depth of focus and requires simple sample preparation operation, and is suitable for analysis of sample sizes from nm to cm. Ko et al (2012) produced allyl isothiocyanate microcapsules by spray drying; SEM was employed to characterize the morphology of the particles and the spherical shape, surface dents and shrinkage were clearly observed.[6] In another study, eugenol was used as a model compound for encapsulation in conjugates of whey protein isolate and maltodextrins made from different mass ratios, the microcapsules demonstrated mostly spherical and micrometer-sized particles with some collapsed structures by SEM.[4] The flavor microcapsules containing vanilla oil were developed using complex coacervation approach, and the SEM analysis revealed the spherical shape and smooth surface of the microcapsules. [7]

For the last few years, extensive studies have been conducted using X-ray micro-CT for investigating internal configuration of objects in various science and engineering fields.[8] It is a nondestructive technique that visualizes the internal morphology of particles based on density differences and therefore it became a commonly used tool in investigating the microstructural behaviors of materials. Micro-CT was used to investigate the three dimensional distribution of the built-in (micro-) capsules of an autonomic self-healing materials, and the breakage and leakage behavior of (micro-) capsules at the micrometer scale was elucidated.[9] Also,

poly(phenol-formaldehyde) microcapsules that aim to provide a self-healing function for cementitious materials were prepared and the rupture behavior and crack surface of cement paste with embedded microcapsules were observed and analyzed using micro-CT.[10] Although micro-CT characterization in flavor microcapsules was not commonly seen, it was considered as an appropriate tool to indicate the internal distribution of 2AP-$ZnCl_2$ complex in microcapsules in this study due to the great density difference between the complex and the wax.

Quantitation of flavor loading is essential to characterize the chemical property of microcapsules since it is a direct reflection of the effectiveness of adopted encapsulation approach. GC is a widely used and accurate method for compositional analysis for flavor microcapsules.[11–15] For example, Ko et al. (2012) measured total allyl isothiocyanate retention by dispersing the encapsulate powder in distilled water, followed by the addition of diethyl ether with phenyl isothiocyanate as an internal standard (I.S.); the organic phase and water were then centrifuged to separate the layers, and the flavor content of the organic phase was measured by GC-The highest retention reached was 84.66%.[6] In this study, the flavor loading in microcapsules was measured by GC; and the basic analytic steps for the 2AP content determination includes dissolving the wax layer, releasing the free flavor molecule upon hydration of the complex, and direct extraction of active compound by a solvent containing the labelled $^{13}C_2$-2AP as I.S.. Meanwhile, absorbance spectroscopy is considered as a low cost and simple technique for compositional analysis[7]; it was therefore used to measure the $ZnCl_2$ content in microcapsules.

5.3 Materials and Methods

5.3.1 Materials

2AP-$ZnCl_2$ microcapsules were prepared by spray chilling encapsulation technique by use of paraffin wax (octacosane) as the wall material, as described in chapter 4. The microcapsules possessed free flowing properties for more than 6 months when stored at ambient dry conditions in a vial equipped with a PTFE-lined silicon cap, prior to physical and chemical characterization. Labelled $^{13}C_2$-2AP was synthesized following the 2AP synthetic route described in chapter 2, by replacing potassium cyanide and iodomethane with ^{13}C labelled reagents. Zinc dust (<10 μm) and zincon monosodium salt were purchased from Sigma-Aldrich (St Louis, MO, USA). Boric acid, sodium borate, 36.7% (w/w) HCl, sodium hydroxide, anhydrous diethyl ether, sodium sulfate and 2 ml GC vials were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

5.3.2 Scanning electron microscopy (SEM)

SEM technique was used to evaluate the surface morphology of the microcapsules. The characterization was performed in the Microscopy Suite of Beckman Institute at the University of Illinois at Urbana-Champaign. An environmental scanning electron microscope (ESEM) with a field-emission electron gun (XL30 ESEM-FEG; Philips/FEI Co., Hillsboro, OR, USA) was used and operated in high-vacuum mode at 5 kV, spot 3 (2.1 nm). A small amount of microcapsules was placed in the center of a 25 mm diameter carbon double-stick tab attached to the aluminum stub. To aid conductivity, the particles were then pressed evenly into the carbon tab using nonstick release paper. The stub was then coated with ca. 7 nm of gold-palladium using

a Denton Vacuum Desk-2 turbo sputter coater (Moorestown, NJ, USA). Images were collected at various magnifications and the particle size distribution was estimated.

5.3.3 X-ray micro-CT

The X-ray micro-CT scanning of 2AP-$ZnCl_2$ microcapsules was applied to investigate the internal structure of the particles. The 2D image was performed by a high-resolution X-ray imaging system Xradia MicroCT (MicroXCT-200) (Carl Zeiss X-ray Microscopy Inc., Pleasanton, CA, USA) with sub-1 micron pixel resolution. It was operated at powder source of approximately 10 W at 40 kV with 1 sec of exposure time. The microcapsules were mounted for scanning by adhering a thin layer of particles to a 2 mm wide and 1 cm long rectangular piece of scotch tape.

A high-resolution 3D X-ray imaging system Xradia MicroCT (MicroXCT-400) (Carl Zeiss X-ray Microscopy Inc., Pleasanton, CA, USA) was used for 3D scanning. The system had a 4×magnification lens and power source of approximately 8 W at 63 kV and 127 μA with 1 sec of exposure time. The rotating angle was from -180 to 180 that generated 955 image slices with a voxel size of 3.34 × 3.34 × 3.34 μm. The microcapsules sample was sealed in a 1 cm long transparent polyethylene tube, prior to scanning.

5.3.4 Determination of 2AP content in microcapsules

Approximately 10 mg of 2AP-$ZnCl_2$ microcapsules were used for 2AP content (flavor loading) determination. An etheric $^{13}C_2$-2AP solution at a concentration of 16.25 ng/μl was

prepared as internal standard (I.S.) solution. The weight of the microcapsules were carefully recorded in a 2 mL GC vial by an analytical balance, 0.5 mL of I.S. solution was added to the vial in order to dissolve the wall material of the particles, the mixture was shaken by hand for exact 1 min before being centrifuged at 3000 RPM for 5 min. The upper ether layer was collected from the vial and dehydrated with sodium sulfate, prior to GC analysis. Quantitation was done by comparing peak areas ratio for unlabelled 2AP (ion 111) and $^{13}C_2$-2AP (ion 113) by use of a response factor of 1.03, specific calculations was followed by equation 3.

$$\text{2AP content (\%)} = \frac{\frac{Peak\ \ area\ \ of\ \ unlabelled\ \ \ 2AP\ \ (111)}{Peak\ \ area\ \ of\ \text{labelled}\ \ \ 2AP\ \ (113)} \times concentration\ \ \ of\ I.S.(mg/mL) \times 0.5\ mL \times R_f}{Weight\ of\ mirocapsules\ \ \ (mg)} \times 100\% \quad (3)$$

5.3.5 Determination of $ZnCl_2$ content in microcapsules

Approximately 5-10 mg of microcapsules was weighed in 2 mL glass vial following by the addition of 0.5 mL of ether and 0.5 mL of water (pH 2), subsequently. The mixture was then vortexed for 1 min followed by centrifugation (5 min at 3000 RPM). The ether layer was decanted (discarded) and any residual ether remaining in the aqueous phase was removed by purging with nitrogen gas. The aqueous phase was diluted to 1 mL in a volumetric flask and 70 µL aliquot of this solution plus 100 µL of Zincon dye solution [1.6 mM; consisting of 51 mg of Zincon sodium salt in 1 mL of NaOH (1M) then diluted to 50 mL with water] and 3 mL of borate buffer (50 mM, pH 9.0) were combined in a cuvette. After 5 min incubation at room temperature, the absorbance was measured at 620 nm using a Lambda 1050 UV/vis/NIR spectrophotometer (PerkinElmer Inc., Shelton, CT, USA). A 6-point calibration curve was constructed from a

dilution series of a zinc stock solution [107 mg of zinc dust dissolved in 1 mL of 36.7% (w/w) HCl for 4 h prior to dilution to 1 L].

5.3.6 Gas chromatography

GC was performed using a 6890N GC/5973N mass selective detector (MSD; Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a fused-silica capillary column (Stabilwax, 30 m × 0.25 mm × 0.25 μm film thickness; or Rtx-5, 15 m × 0.32 mm × 0.5 μm film thickness; Restek, Bellefonte, PA, USA). Helium was the carrier gas at a constant flow of 1 mL/min. The injector was held at 250°C in the splitless mode. GC oven temperature was programmed from 40 to 225 °C at 10 °C/min with initial and final holding times of 5 and 40 min, respectively. MS was operated in the EI-SIM mode. Other conditions for the MSD were as follows: MSD interface temperature, 260°C; ionization energy, 70 eV; mass range, 35-350 amu; EM voltage, Autotune +165 V; scan rate, 4.45 scans/s. Quantitation was done using area ratio, mass ratio and response factor as previously described.

5.4 Results and Discussion

5.4.1 Characterization of 2AP-$ZnCl_2$ microcapsules by SEM

The morphology of the microcapsules is of importance since it is directly related to the physical properties such as flowability and flavor release characteristics. Microcapsules made with 2AP-$ZnCl_2$ and octacosane wax were studied by SEM. Spherical shape of our product is shown in Figure 5.1, which is a common characteristic for encapsulation products. The surface

of the microcapsules is overall smooth except for a few brittle flakes attached; which were residues from the wall material octacosane. No obvious ruptures and crackers were observed from the SEM graphs, which indicated the surface integrity of the microcapsules. It was considered as an excellent characteristic compared to some studies which reported cracking particles[4,6], because less flavor loss was expected for intact microcapsules during transportation and storage. Also, the particle size is an important characteristic and the preferred size range for microcapsules in food applications is 20-200 μm.[16] Although a wide range of particle size distribution is observed, SEM graph indicates the dimeters of the microcapsules range from approximately 5 to 200 μm; therefore, our product was considered satisfactory in terms of particle size. Overall, the wax complex spray-chilling system produces microcapsules with desirable morphological characteristics (spherical shape, smoothness and particle size).

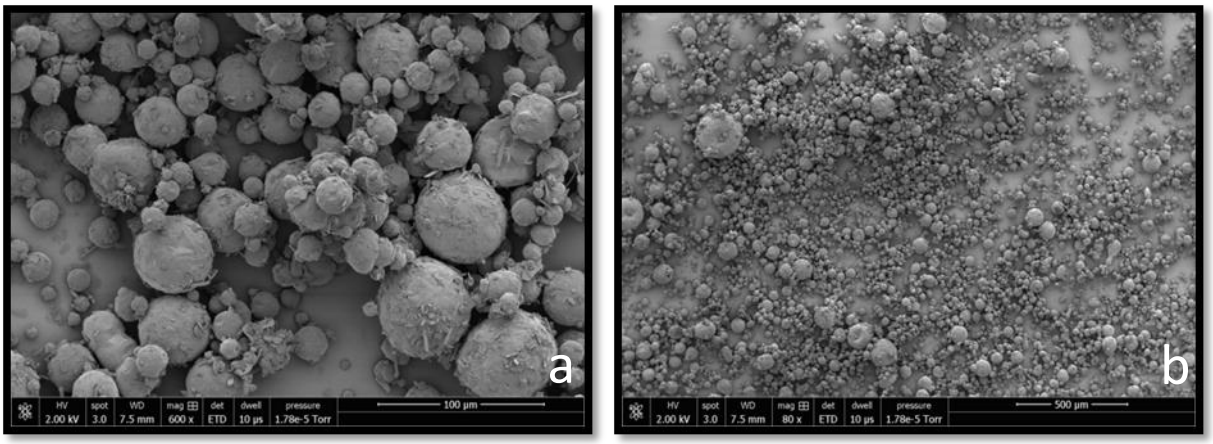

Figure 5.1 SEM graph of 2AP-$ZnCl_2$ octacosane microcapsules containing a flavor loading of (0.0814±0.0048) % (mean±SD, n=3) at (a) 600×magnification and (b) 80×magnification.

5.4.2 Micro-CT characterization

Micro-CT is a technique which provides images of the internal structure of an object based on density differences. The X-ray transmission image of the 2AP-$ZnCl_2$ microcapsules is shown in Figure 5.2. The spherical shape of the microcapsules was distinguished from the background. Within the particle, the black dots indicating the complex can be readily distinguished from the grey continuous wax phase. This contrast is due to the density differences between the core (higher density complex, above 1.33 g/mL from lab experimental data) and wall materials (lower density wax, 0.81 g/mL, according to the wax specification sheet provided by the manufacturer). Therefore, the internal structure and complex distribution was revealed.

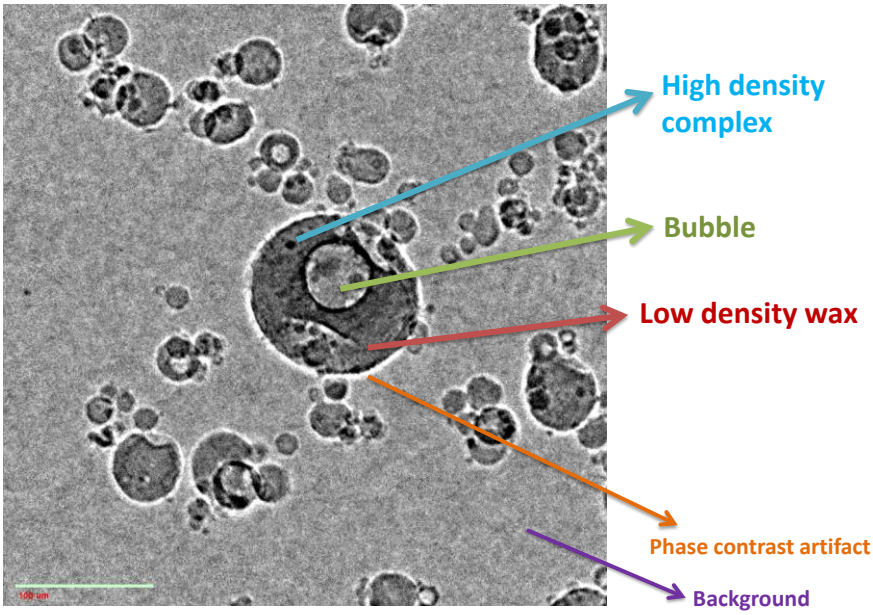

Figure 5.2 The X-ray transmission images of the 2AP-$ZnCl_2$ microcapsules with a flavor loading of (0.0814±0.0048) % (mean±SD, n=3).

However, in order to confirm the true encapsulation of complex within the wax material, 3D micro-CT scanning was applied. Time series of 3D image slicing of 2AP-$ZnCl_2$ microcapsules was compiled; the complex firstly appeared in Figure 5.3 (a)(b) at the arrow pointed region, and the area of the black scope (high density complex) increased as slicing continued [Figure 5.3 (c) to (f)], and then gradually disappeared [Figure 5.3 (g) to (i)]. This phenomenon/change confirmed that the active complex was well encapsulated by the octacosane wax. Moreover, 2AP-$ZnCl_2$ product were validated to be a matrix type of microcapsules by use of SEM and Micro-CT techniques; which was in agreement with other studies which indicated that spray chilling encapsulation produces matrix type microcapsules.[17] Although micro-CT is an emerging technique for investigation of internal configurations of objects, with extensive applications in materials science area to study the three dimensional distribution of microstructures[9,10]; no study was found to apply this technique in characterization of flavor microcapsules before. This work firstly demonstrated the practicability of using micro-CT as a useful tool to evaluate the internal structure of flavor microcapsules.

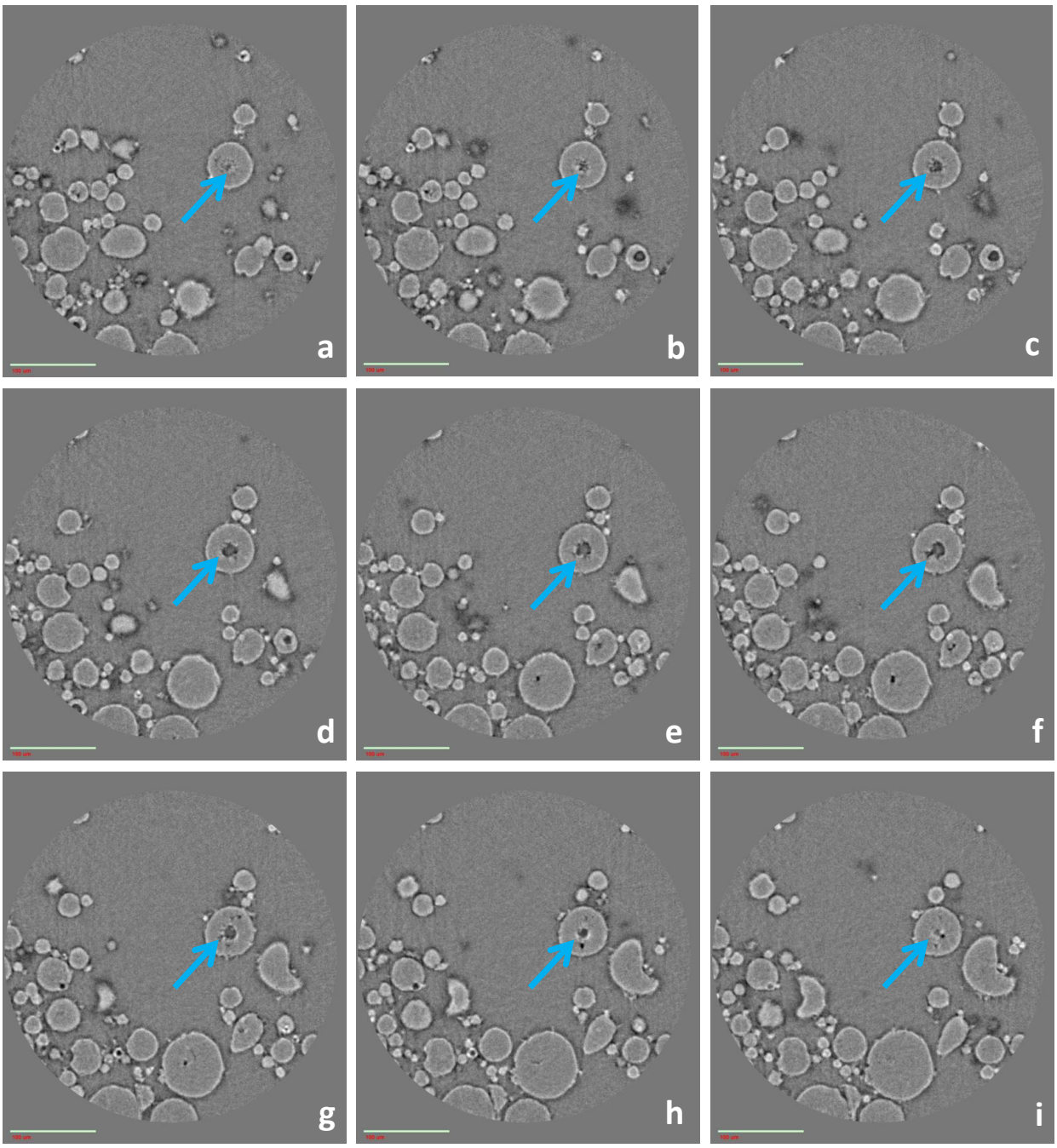

Figure 5.3 Time series of 3D image slicing of 2AP-$ZnCl_2$ microcapsules with a flavor loading of (0.0814±0.0048) % (mean±SD, n=3).

5.4.3 Determination of 2AP and $ZnCl_2$ contents in microcapsules

The 2AP and $ZnCl_2$ contents in the microcapsules were measured. With the 2AP loading of 0.0814% and $ZnCl_2$ contents of 0.3681%, the mass ratio of the two stayed consistent during the spray-chilling process. The results revealed that no degradation of the 2AP-$ZnCl_2$ complex was caused by this encapsulation technique; it also confirmed the appropriateness of above two testing procedures by showing good reproducibility of data.

5.5 Conclusion

The chemical and physical properties of the 2AP-$ZnCl_2$ microcapsules were characterized by proper testing methods. SEM and Micro-CT were found to be good tools to study the morphology, internal structure and flavor complex distribution in spray-chilled microcapsules. Methods for accurately measuring 2AP and $ZnCl_2$ contents were developed. The microcapsules exhibit desirable characteristics for food application.

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Chapter 6

INVESTIGATION OF 2-ACETYL-1-PYRROLINE STABILITY IN MICROCAPSULES DURING STORAGE

6.1 Abstract

The stability of 2AP in pure $ZnCl_2$ complex as well as in microcapsules was determined by use of a time course study. Three relative humidity (RH) conditions (~0%, ~22.5% and ~43.2%) at ambient temperature were chosen for microcapsules and complex storage studies. By monitoring the changes of 2AP in both forms using gas chromatography (GC), its stability was determined. The results showed that, at all storage conditions, the microcapsules retained significantly improved 2AP stability comparing to the unprotected complex. The microcapsules were shown to have the potential for commercialization due to its high 2AP stability for more than 3 months storage at ambient temperature, as long as they are packaged in a moisture-barrier bag.

6.2 Introduction

Protecting the loss of flavor compounds during storage, controlling the release of the flavors during food processing or end use and promoting easier handling are three main objectives for flavor encapsulation.[1,2] The stability of encapsulated flavors is important for estimating the shelf-life of the flavor and for controlled release applications in food.[3] The storage RH and temperature generally affect the flavor release from the microcapsules. Therefore, flavor

stability should be assessed at various storage conditions in order to gain more understanding of appropriately handling and for applying the microcapsules.

Although several relative humidity conditions were selected during the storage course, only room temperature was considered since the final microcapsules would only be applicable if they show improved stability at ambient conditions. 2AP-$ZnCl_2$ microcapsules have a wax coating and flavor release is triggered by heat-induced melting of this coating. Since 2AP molecule could be found in almost all cooked or heated food, the obtained microcapsules in this study could be potentially applied in convenience foods and bakery goods such as instant rice, noodle soup and bread dough.

Besides ambient dry condition (~0%), two saturated salt solutions potassium acetate (CH_3COOK) and potassium carbonate (K_2CO_3) were selected to create comparable RH levels at room temperature, based on the survey of water activity values (a_w) of above mentioned foods (~0.2 and ~0.4).[4] A time-course study of 2AP stability in both complex and microcapsules under three ambient storage conditions were investigated. Changes in 2AP content as a function of storage period was measured by gas chromatography (GC).

6.3 Materials and Methods

6.3.1 Materials

2AP-$ZnCl_2$ microcapsules were prepared by spray chilling encapsulation technique by use of paraffin wax (octacosane) as the wall material, as described in chapter 4. The microcapsules possessed the free flowing properties before subjected to storage studies.

Phosphorus pentoxide, potassium acetate and potassium carbonate were purchased from Sigma-Aldrich (St Louis, MO, USA). Anhydrous diethyl ether, methylene chloride and sodium sulfate were ordered from Fisher Scientific (Fair Lawn, NJ, USA). 2AP-$ZnCl_2$ complex was also used for storage as a control to compare with microcapsules. It was made through complexation of 2AP with $ZnCl_2$ solution followed by centrifugation and nitrogen purge, as described in chapter 3. Before storage study, the complex was kept dry at -20°C in a vial equipped with a PTFE-lined silicon cap. Labelled $^{13}C_2$-2AP was synthesized following the 2AP synthetic route described in chapter 2, by replacing potassium cyanide and iodomethane with ^{13}C labelled reagents. An etheric $^{13}C_2$-2AP solution at a concentration of 16.25 ng/µl was prepared as internal standard (I.S.) solution.

6.3.2 Storage study setup

The whole storage study was set at ambient temperature conditions, in order to assess the practicality of using 2AP-$ZnCl_2$ microcapsules in food industry. The microcapsules could be potentially used as a flavoring agent to instant rice and baking mix; therefore, the RHs of these foods were considered for this study. Table 6.1 includes the water activity (a_w) values of a list of potential food matrices that could employ 2AP-$ZnCl_2$ microcapsules as a flavoring agent. Their a_w values indicate the preferred RHs for designing storage conditions for microcapsules. Therefore, two saturated salt solutions in Table 6.2 were selected to create preferred storage conditions for microcapsules. Besides storage under desiccated conditions by phosphorus pentoxide (P_2O_5) to create a near 0% RH, saturated CH_3COOK and K_2CO_3 solutions were

selected to maintain the RH at ~22.5% and ~43.2% at room temperature, respectively, according to ASTM Standard E104-02 (Reapproved 2012)[5].

Saturated salt solutions were prepared in 20 mL scintillation glass vials, following the detailed procedure indicated in ASTM Standard E104-02, according to the solubilities of CH_3COOK and K_2CO_3 in water. For each replicate, the microcapsules (5-10 mg) made from 2AP-$ZnCl_2$ and octacosane were stored in a loosely-caped 2 mL GC vial; the GC vial was then placed in a 20 mL vial containing saturated salt solution which was sealed well with a PTFE-lined silicon cap 24-400 [Figure 6.1 (a)]. For replicates under desiccated conditions, free flowing P_2O_5 powder was used instead of saturated salt solution [Figure 6.1 (b)]. The storage of microcapsules under dry condition lasted for more than 3 months, while the others were done in three weeks. The 2AP-$ZnCl_2$ pure/unprotected complex was stored under the same conditions as the microcapsules to serve as the control. The 2AP stabilities in complex and microcapsules were monitored during storage.

Table 6.1 Water activity values of selected foods and food ingredients.

Item	**a_w value**	**Temp (°C)**	**Source**
All-purpose flour, Gold Medal	0.453	20	4
Corn starch, Argo 100%	0.287	20	4
Buttermilk baking mix, Bisquick	0.436	25	Lab data
All-purpose baking mix, Jiffy	0.460	25	Lab data
Long grain instant white rice, Meijer	0.269	25	Lab data
Long grain instant white rice, Uncle Ben's	0.195	25	Lab data

Table 6.2 Storage conditions for 2AP-$ZnCl_2$ microcapsules at room temperature.

Item	RH value	Temp (°C)	Source
Phosphorus pentoxide (P_2O_5)	~0%	RT[a]	Lab data
Saturated potassium acetate solution (CH_3COOK)	~22.5%	RT	ASTM Standard E104-02, 2012
Saturated potassium carbonate solution (K_2CO_3)	~43.2%	RT	ASTM Standard E104-02, 2012

[a]RT: room temperature, generally ranges from 22 to 25°C.

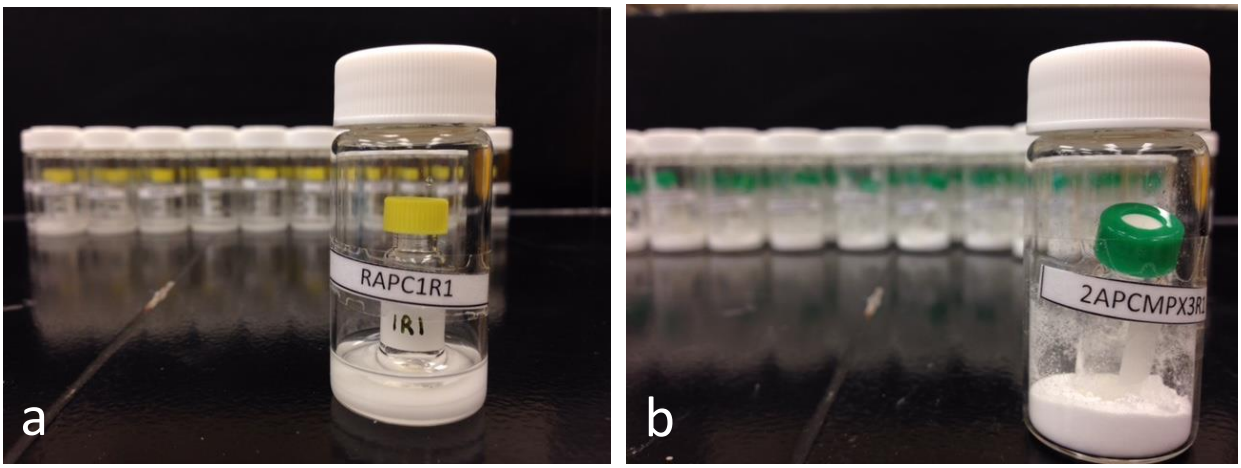

Figure 6.1 Storage conditions controlled by (a) saturated salt solution (~22.5% or ~43.2% RH) and (b) P_2O_5 powdered desiccant (~0%) for a single replicate.

6.3.3 Monitoring 2AP stability in microcapsules and complex

The 2AP stability in both microcapsules and complex were monitored by measuring the changes in 2AP content within the storage period; it was calculated by dividing the 2AP content at each storage point by its initial content prior to storage.

Procedure of 2AP content determination in microcapsules was followed as described in chapter 5. 2AP content in $ZnCl_2$ complex was determined according to the method developed by

Fang and Cadwallader (2014)[6], with a few modifications. Approximately 2-5 mg of complex was placed and accurately weighed in a 2 mL vial, 0.5 mL of phosphate buffer (50 mM, pH = 7) was added to free the 2AP molecule, then 0.5 mL methylene chloride containing I.S. $^{13}C_2$-2AP (16.25 ng/µl) was added to the mixture and the extraction was fulfilled by vigorously shaken by hand for 1 min, prior to centrifugation at 3000 RPM for 5 min. The lower solvent layer was then collected from the mixture and dried with sodium sulfate before GC anlysis. Quantitation of 2AP content in complex was done by comparing peak areas ratio for unlabelled 2AP (ion 111) and $^{13}C_2$-2AP (ion 113) by using a response factor of 1.03, the calculations followed equation 2 in chapter 5, by replacing the denominator by the weight of the complex.

6.3.4 Gas chromatography (GC)

GC was performed using a 6890N GC/5973N mass selective detector (MSD; Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a fused-silica capillary column (Stabilwax, 30 m × 0.25 mm × 0.25 µm film thickness; or Rtx-5, 15 m × 0.32 mm × 0.5 µm film thickness; Restek, Bellefonte, PA, USA). Helium was the carrier gas at a constant flow of 1 mL/min. The injector was held at 260°C in the split mode for measurement of complex and splitless mode for microcapsules measurements, respectively. For hot split mode, the GC oven temperature was programmed from 40 to 225 °C at 10 °C/min with initial and final holding times of 5 min; while the final holding time for hot splitless mode was extended to 40 min. MS was operated in the EI-SIM mode. Other conditions for the MSD were as follows: MSD interface temperature, 260°C; ionization energy, 70 eV; mass range, 35-350 amu; EM voltage, Autotune

+165 V; scan rate, 4.45 scans/s. Quantitation was done using area ratio, mass ratio and response factor as previously described.

6.4 Results and Discussion

6.4.1 2AP stability in microcapsules and complex in desiccated condition

The stability of 2AP was studied over a period of 3 months and the data is shown in Figure 6.2. Under desiccated conditions, the microcapsules exhibited significantly improved 2AP stability, compared to pure complex. The stability of 2AP in microcapsules and complex forms were 69.65% and 45.91%, respectively, after 3 month storage at ambient dry condition. Therefore, among those two forms, the microcapsules are more competitive to be commercialized as a flavoring agent since it obviously possesses a longer shelf life. Meanwhile, the wax layer of the microcapsules were, as expected, verified to be an effective moisture barrier to avoid or at least delay the degradation progress of 2AP-$ZnCl_2$ complex to some extent. In previous studies, 2AP was encapsulated in many forms to improve its stability. Freeze-dried 2AP microcapsules were obtained by wall materials maltodextrin and β-cyclodextrin, the 2AP degradation was over 90% after 13 days' storage at 20°C; the RH condition, however, was not specified.[7] Gum acacia and maltodextrin mixtures were used as wall materials to encapsulate 2AP in aqueous solutions by spray-drying, the degradation of 2AP in microcapsules ranged from 27.7% to 43.2% after 72 days of storage at well-sealed ambient conditions; however, the 2AP loading in the spray-dried microcapsules was too low (0.003%) for food application.[8] An ethanol solution of 2AP was homogenized with wall materials gum acacia and starch before vacuum shelf drying or spray-drying to form free flowing microcapsules; however, neither storage nor

stability data was available.[9] Additionally, a pandan extract comprised high amount of 2AP was spray-dried with maltodextrin and β-cyclodextrin to form microcapsules, the stability data was again not available.[10] Therefore, to the best of our knowledge, the results here represent the best 2AP stability after long-term ambient storage and are the most applicable one for food applications, among all the previous studies.

A dramatic decrease of 2AP stability in both forms was observed in the first two weeks of storage. Possible reasons for this phenomenon included the following: 1) 2AP-$ZnCl_2$ complex was incompletely encapsulated and the free complex attached to the surface of the microcapsules was more prone to degradation; 2) the first two weeks might be the equilibration period for the 20 mL vial to reach near 0% RH, but a small amount of water vapor still existed in the system at the beginning of the study which in turn caused the degradation of 2AP. However, almost no pure complex was observed on the surface of microcapsules when characterized by SEM and X-ray micro-CT; therefore, the first reason might not be persuasive. Additionally, a steady trend of 2AP stability was observed during the storage after 2 weeks, which suggested the possibility of the microcapsules having an even longer shelf life than 3 months with acceptable 2AP stability. Furthermore, P_2O_5 was proved to be an excellent desiccant to create a ~0% RH in a 20 mL well-sealed system.

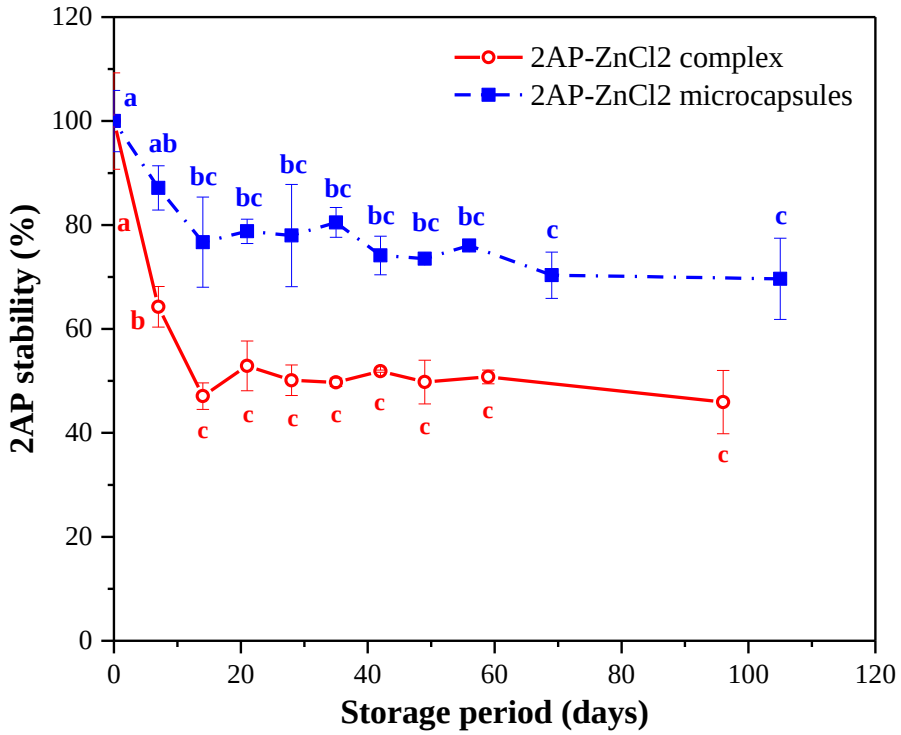

Figure 6.2 Stability of 2AP-$ZnCl_2$ complex in its pure (unprotected) and microcapsules forms stored in a well-sealed vial containing desiccant at room temperature (Mean±SD, n=3). Different letters having the same color text indicate significant sample differences as a function of storage period ($p < 0.05$).

6.4.2 2AP stability in other RH conditions

2AP stability in both 2AP-$ZnCl_2$ complex and microcapsules were evaluated at two selected RH levels (~22.5% and ~43.2%) at room temperature. At both RHs, the stability of 2AP in microcapsule form was significantly increased comparing to unprotect complex (Figure 6.3); which again indicated that the wax wall effectively retarded the degradation of the complex caused by moisture invasion. Although the 2AP stability in microcapsules kept at ~43.2% condition retained above 10% after 3 days of storage, much higher comparing to pure complex (dropped below 2% after 2 days of storage); storing the microcapsules under such level of RH

was not recommended to maintain high 2AP stability. A separate moisture-preventing packet might be adopted to preserve the microcapsules as a flavoring agent during shelf life.

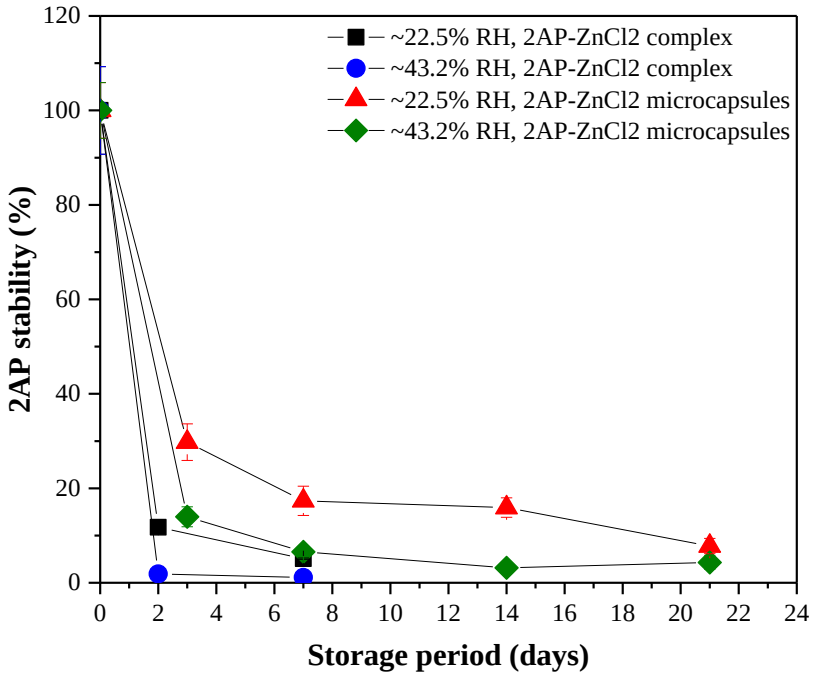

Figure 6.3 Stability of 2AP-$ZnCl_2$ complex in its pure (unprotected) and microcapsules forms stored in a well-sealed vial containing saturated salt solutions to create ~22.5% and ~43.2 RHs, respectively (Mean±SD, n=3). Different letters having the same color text indicate significant sample differences as a function of storage period ($p < 0.05$).

6.5 Conclusion

The 2AP stability in microcapsules was significantly improved compared to the unprotected complex, at all ambient storage conditions (0%, ~22.5% and ~43.2% RH). In order to retain the maximal stability, 2AP microcapsules could be commercialized by use of a single packet (much like a ~0% RH condition) as a flavoring agent for food industry. Noticeably,

microcapsules are much easier to be transferred and handled at regular atmosphere with no degradation; which is a very welcomed feature for real application. Storage of 2AP-$ZnCl_2$ microcapsules at an elevated temperature such as 35 or 40°C could be included in the future research, in order to investigate its stability during transportation and distribution of commercialized products.

6.6 References

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Chapter 7

APPLICATION OF 2-ACETYL-1-PYRROLINE ZINC CHLORIDE MICROCAPSULES AS A CONTROLLED RELEASE FLAVORING AGENT IN FOODS

7.1 Abstract

This chapter describes the first application of 2AP-$ZnCl_2$ microcapsules in real foods, and demonstrates the feasibility of using the microcapsules as an effective flavoring agent since high flavor recovery (almost 100%) was achieved during the cooking process of instant rice. The flavor recovery was measured by quantitation of free 2AP released to the headspace of the sealed cooking system. The pure 2AP-$ZnCl_2$ complex was also evaluated under the same cooking conditions to serve as the comparison to the microcapsules. The microcapsules have several advantages over the use of the pure complex, including the possibility of controlled release and high 2AP recovery as well as enabling easier material handling and transfer practices.

7.2 Introduction

Besides improving chemical and physical stability of the core material, the potential for controlled release is another major function of microcapsules.[1] Release of flavor continues to be the key criterion for selecting an appropriate encapsulation system and the well-controlled release of an active is critical in various applications.[2] Enabling the release of flavor at the right

place and time are often desired. The release mechanisms fit into several categories: release by physical rupture, by diffusion, by dissolution or melting and by biodegradation.[2]

Since the moisture sensitive/degradable 2AP-$ZnCl_2$ complex is successfully encapsulated with a moisture barrier, the microcapsules are expected to maintain 2AP-$ZnCl_2$ complex stability throughout the application steps prior to heating. During heat processing or cooking of foods, the wax material will be melted, allowing the complex to be exposed to moisture, and thus resulting in the release of the free 2AP molecules. In this study, instant rice was chosen as the food matrix for application study of the microcapsules for two reasons: 1) flavoring bland instant rice with 2AP should greatly improve its sensory properties and consumer acceptance; and 2) the flavor complex will undergo controlled release from the microcapsules after wax coating is melted during the rice cooking process, thus maximizing the release of 2AP in the final product.

There are several methods to characterize the flavor release from microcapsules. One of them is the determination of the volatiles released to the headspace.[3,4] In this study, instant rice was selected to investigate the feasibility of using the 2AP-$ZnCl_2$ microcapsules as a controlled release flavoring agent, and the measurement of flavor recovery was implemented by static headspace solid-phase microextraction (HS-SPME) and gas chromatography (GC) analysis.

7.3 Materials and Methods

7.3.1 Materials

Microcapsules were produced from octacosane and 2AP-$ZnCl_2$ complex by spray-chilling encapsulation technique as described in chapter 4. The microcapsules possessed the

desired free flowing property before use. 2AP-$ZnCl_2$ complex was made through complexation of 2AP with $ZnCl_2$ in an etheric solution followed by washing, centrifugation and nitrogen purge, as described in chapter 3. The synthesis protocol of internal standard (I.S.) $^{13}C_2$-2AP was described in chapter 2. A methylene chloride solution containing 1.625 µg/µl of $^{13}C_2$-2AP was prepared and used as I.S. solution. Monopotassium phosphate (KH_2PO_4), dipotassium phosphate (K_2HPO_4) and methylene chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA) to prepare the phosphate buffer (50 mM, pH 6.4). Minute® instant rice (enriched long grain white rice, Riviana Foods Inc., Houston, TX, USA) was purchased at a local supermarket. Corn starch was purchased from EM Science (Gibbstown, NJ, USA), and was baked at 130°C for 6 h prior to use.

7.3.2 Procedure for application study

Instant rice was chosen as the food matrix for this application study. The microcapsules, with a 2AP loading of 0.05% (w/w), were used as the flavoring agent. Assuming a high flavor recovery, 10 mg of microcapsules with this loading would provide approximately 1000 ppb (ng/g) to the cooked rice, which is within the preferred range of 2AP content in aromatic rice.[5,6] Figure 7.1 shows the main steps of microcapsules cooking with instant rice. The weight of approximately 10 mg of free-flowing 2AP-$ZnCl_2$ microcapsules was recorded and added to 5 g of instant rice in a 50 mL vial under ambient conditions (i.e., without creating a near 0% relative humidity (RH) environment for transfer). After addition of 10 mL of odorless water, the vial was sealed with a PTFE-lined cap and then heated by submerging the sealed vial in a boiling water bath for 5 min. The vial [Figure 7.1 (a)] was then cooled in ice water bath for 5 min then spiked

with 3 µl of $^{13}C_2$-2AP (1.625 µg/µl) as I.S. Subsequently, 30 mL odorless water was added to the vial, then it was sealed and mixed well [Figure 7.1 (b)], before incubation at 60°C for 20 min. The flavor/2AP extraction from the sealed vial was performed by static headspace solid-phase microextraction (HS-SPME). A 1 cm 50/30 divinylbenzene/carboxen/PDMS fiber (Supelco, Bellefonte, PA, USA) was exposed to the headspace of the vial for an additional 20 min at 60°C to facilitate adsorption of 2AP and the I.S. [Figure 7.1 (c)]. Controls consisted of 10 mg of the microcapsules plus 10 mL phosphate buffer (50 mM, pH 6.4) followed by cooking and analysis as described above [Figure 7.1 (d)]. Flavor recovery was calculated by dividing the determined 2AP amount from cooked rice with the 2AP amount recovered from buffer control [equation (4)]. The above treatments were done in triplicate.

$$Flavor\ recovery = \frac{2AP\ recovered\ \ from\ cooked\ rice}{2AP\ recovered\ \ from\ buffer\ \ control} \times 100\% \quad (4)$$

Besides the microcapsules, a mixture of 2AP-$ZnCl_2$ complex and dry starch (with a flavor loading of 0.05%) was also cooked with instant rice or phosphate buffer in order to compare the flavor recovery between microcapsules and pure complex. However, handling the complex required special conditions, such as weighing the complex and performing all of the the mixing steps in a lab scale dry chamber (Cleatech Two-Port Portable Mini Glovebox, Santa Ana, CA, USA) filled with nitrogen at an RH lower than 5%.

7.3.3 Gas chromatography (GC)

Volatile compounds were desorbed (260°C; splitless, 4 min valve-delay) from the SPME fiber into a 6890/5973N GC-MS (Agilent Technologies, Inc., Palo Alto, CA, USA) using a Stabilwax column (30 m x 0.25 mm i.d. x 0.25 µm film) with helium (1 mL/min) as carrier gas. The GC oven temperature was programmed from 40 to 225 °C at 10 °C/min with initial and final holding times of 5 and 30 min, respectively. MS was operated in the EI-SIM mode. Quantitation was done by comparing the ratio of the selected ion peak areas for unlabelled 2AP (ion 111) and $^{13}C_2$-2AP (ion 113) by use of a response factor of 1.03.

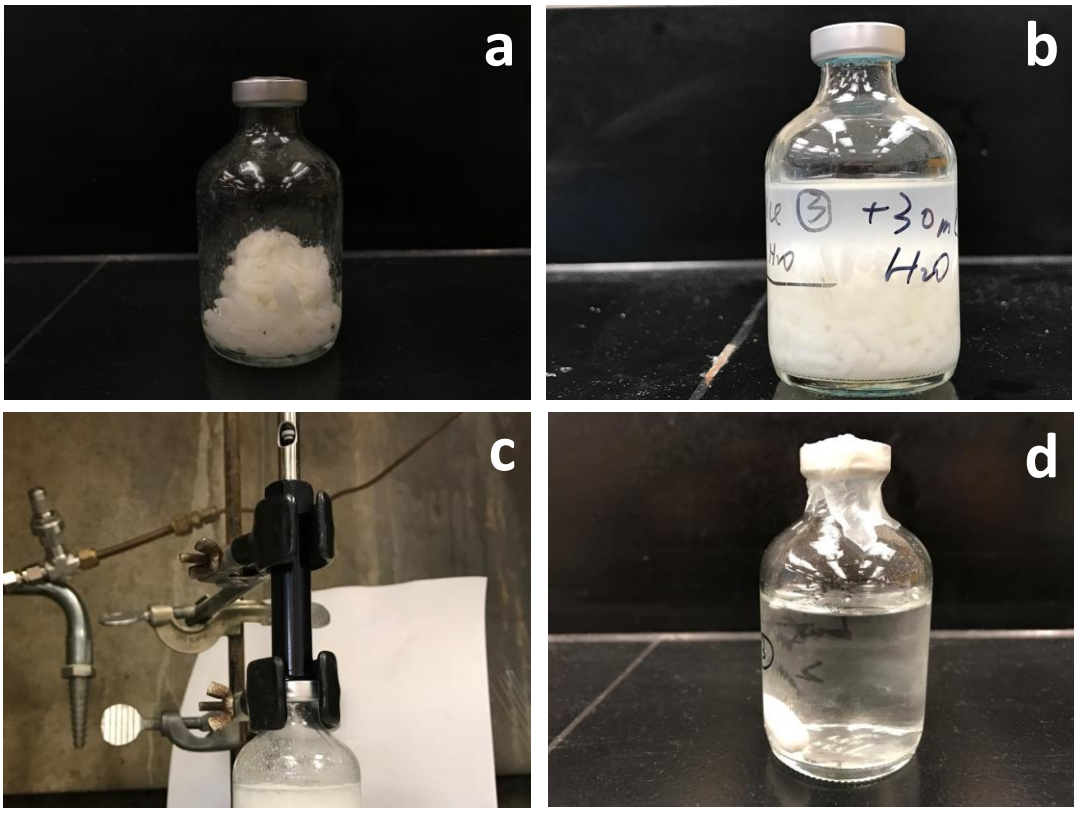

Figure 7.1 Procedure for application of microcapsules in instant rice: (a) 50 mL vial containing cooked rice and microcapsules; (b) 30 mL odorless water added to the vial after spiking $^{13}C_2$-2AP; (c) 2AP extraction by HS-SPME; (d) a control vial containing cooked microcapsules and phosphate buffer.

7.4 Results and Discussion

7.4.1 Flavor recovery of 2AP during rice cooking application

The application of 2AP-$ZnCl_2$ microcapsules as a controlled release flavoring agent to instant rice was investigated by measuring flavor recovery. 2AP recovery from the rice cooking process is presented in Figure 7.2.

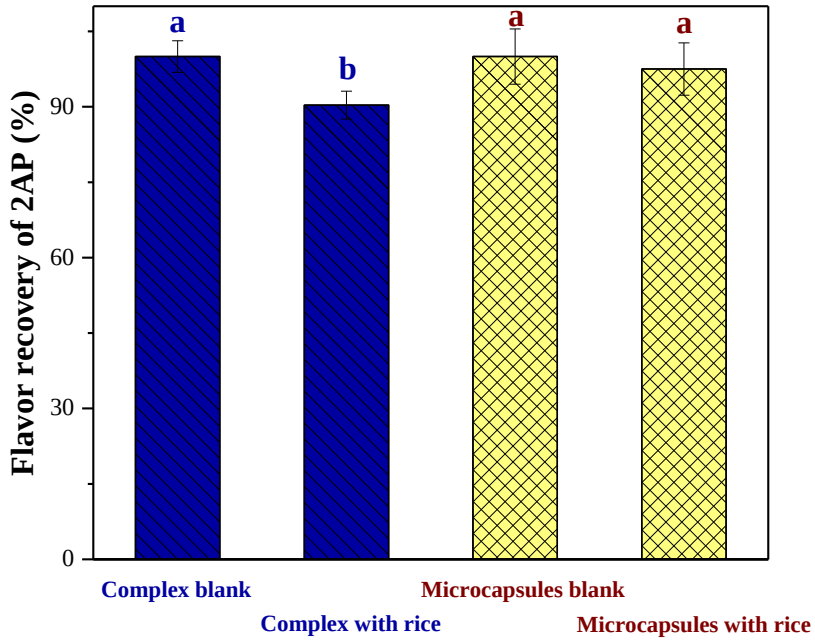

Figure 7.2 2AP recoveries from microcapsules and complex, respectively, after cooking with a real food matrix - instant rice (Mean±SD, n=3). Different letters having the same color text indicate significant sample differences ($p < 0.05$).

Flavor recovery of 2AP from microcapsules cooked with instant rice was almost 100%, since it did not significantly differ from the microcapsules cooked with buffer (the blank/control). Therefore, using 2AP-$ZnCl_2$ microcapsules as a flavoring agent to instant rice, the loss of 2AP during practical application was negligible. Although flavor release from microcapsules largely

depends on the heating temperature in the process and the properties of the food matrix, the controlled release and flavoring properties of produced microcapsules was validated with such a high 2AP recovery. Meanwhile, the 2AP recovery from complex form during cooking process was significantly lower than the control, which indicated the complex experienced some degradation during transfer and cooking, even though it was handled carefully under dry conditions during most of the process.

Although 2AP was discovered 30 years ago and the flavor industry continues the interests in commercializing it into flavoring agents, very few studies reported the 2AP application results in real food matrices. Buttery's group used 0.05 ppm synthetic 2AP aqueous solution to flavor nonaromatic rice and the sensory evaluation showed it successfully provided the "bland" rice having the aroma of "scented" rice variety; however, the flavor recovery was not quantitated and the effectiveness of the 2AP solution as the flavoring agent was not evaluated.[7] In another study, a coating composition manufactured from scented rice containing at least 40 ppb 2AP was added in foods during the cooking or frying process; although an improved sensory profile was reported, neither flavor recovery data nor sensory evaluation results was available to validate the application of the coating composition. Our group reported the use of a mixture of 2AP-$ZnCl_2$ complex and starch powder with a 0.0030% (w/w) 2AP loading to impart an aromatic rice-like character to the instant rice during the cooking process, a high 2AP recovery (92%) was achieved.[8] However, dry condition was required for this practice to ensure the stability of 2AP in complex during transfer and cooking steps, which was not practical for commercialization of this mixture as a flavoring agent. Consequently, the above application of 2AP-$ZnCl_2$ microcapsules in instant rice was the most promising study so far; it not only exhibited full 2AP recovery, but also demonstrated the practical potential of commercializing it as a flavoring agent.

7.5 Conclusion

In this study, the 2AP-$ZnCl_2$ microcapsules were used in a real food matrix as a flavoring agent. The obtained high flavor recovery confirmed its controlled release properties as well as indicated its potential for real food application or even commercialization. The microcapsules possess several advantages over the pure complex: 1) microcapsules could be handled and transferred under various conditions not limited to a dry environment; 2) almost full flavor recovery was achieved by using microcapsules as a flavoring agent for instant rice; meanwhile, some degradation occurred by use of the pure complex; 3) microcapsules demonstrate a controlled release property triggered by heat. Overall, the developed microcapsules were shown to be an effective flavoring agent for instant rice by the analysis of 2AP recovery. Additionally, for future work, the microcapsules could be applied to other matrices which require heat treatment, such as baking products, microwave meals and instant soups.

7.6 References

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Chapter 8

SUMMARY, CONCLUSIONS, IMPLICATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

The goals of this study were to improve the stability of 2AP by chemical and physical approaches and to enable the use of its stabilized form as a flavoring agent in real food matrices. The following goals were achieved: 1) the stability of 2AP was greatly improved by spray-chilling microencapsulation of 2AP-$ZnCl_2$ complex with a layer of paraffin wax; 2) the potential of 2AP-$ZnCl_2$ microcapsules being an effective flavoring agent with controlled release properties was validated in real food application scenarios.

The feasibility of use of the unprotected pure complex to improve the flavor profile of instant rice was investigated under dry conditions; the high recovery of 2AP after the cooking process demonstrated its potential for future application. However, the complex degraded immediately once exposed to moisture; which would hinder its widespread use. Therefore, a moisture barrier would be necessary to maintain its stability prior to use, e.g. during storage and handling.

After modifying the spray-chilling device and selecting appropriate wall materials and aroma complexes, the degradable 2AP-$ZnCl_2$ complex was successfully encapsulated by a layer of paraffin wax (octacosane) as the moisture barrier. The microcapsules were collected as free flowing powder which possessed easy handling and transfer properties.

The chemical and physical properties of the microcapsules were characterized by proper testing methods. SEM and Micro-CT were found to be good tools to study the morphology, internal structure and flavor complex distribution in spray-chilled microcapsules. In addition, methods for accurately measuring 2AP and $ZnCl_2$ contents were also developed. The obtained microcapsules exhibited desirable characteristics for food application.

The storage study showed that the 2AP stability in microcapsules was significantly improved compared to the unprotected pure complex at all ambient storage conditions tested (0%, ~22.5% and ~43.2% RH). In the future, 2AP microcapsules could be commercialized as a flavoring agent by use of a moisture-barrier packet to retain maximal stability. Additionally, microcapsules are much easier to be transferred and handled at regular atmosphere with no degradation; which is a beneficial feature for real application.

Finally, the 2AP-$ZnCl_2$ microcapsules were applied in instant rice as a flavoring agent. The obtained high flavor recovery (almost 100%) confirmed its controlled release properties as well as indicated its potential for real food application or even commercialization. The microcapsules possess several advantages over the pure complex: 1) microcapsules could be handled and transferred under various conditions not limited to a dry environment; 2) almost complete flavor recovery was achieved by using microcapsules from cooking of instant rice; however, some degradation occurred by use of the pure complex; 3) microcapsules feature the controlled release property and could be triggered by heat. Overall, the developed microcapsules were proven to be an excellent and applicable flavoring agent.

For future work, the microcapsules could be applied to other matrices that require heat treatment, like baking products, microwave meals and instant soups, to test its flexibility as a

flavoring agent. Also, the use of wax-complex spray-chilling system could be extended to other aroma complexes such as 2-acetylpyrazine, 2-propionyl-1-pyrroline and 6-acetyl-2,3,4,5-tetrahydropyridine, to solve some existing difficulties with these flavor compounds in food application.

APPENDIX A

Stability of 2AP in complex and microcapsules forms during storage at 100% RH at room temperature

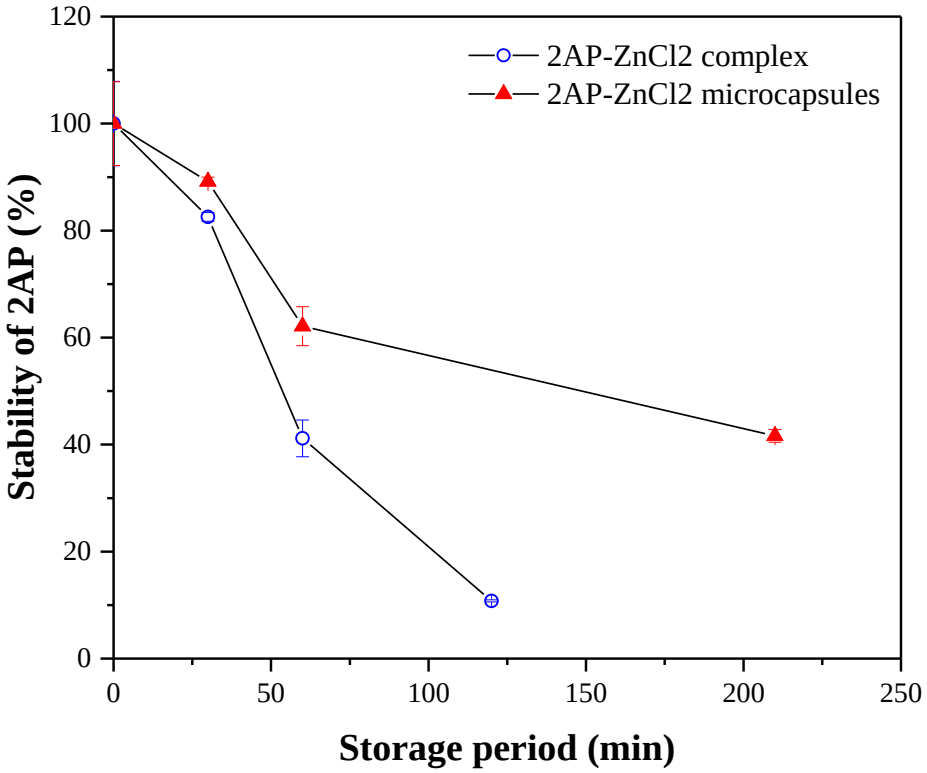

Figure A.1 Stability of 2AP in complex and microcapsules forms during storage at 100% RH at room temperature (Mean±SD, n=3).

APPENDIX B

Physical properties and flavor stability of 2-acetylpyrazine zinc halide (2APra-$ZnCl_2$) microcapsules

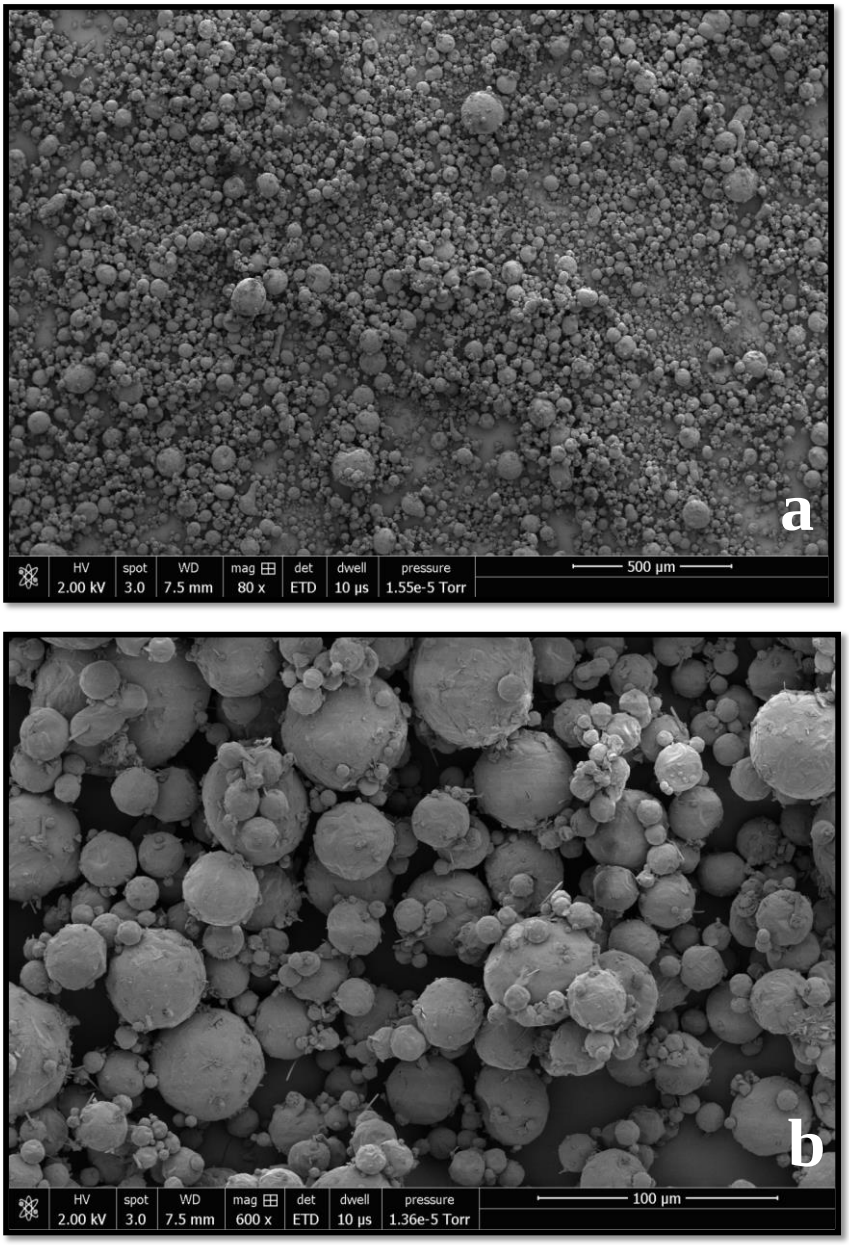

Figure B.1 SEM graph of 2APra-ZnCl2 octacosane microcapsules at (a) 80×magnification and (b) 600×magnification containing a flavor loading of (0.1185±0.0054) % (mean±SD, n=3).

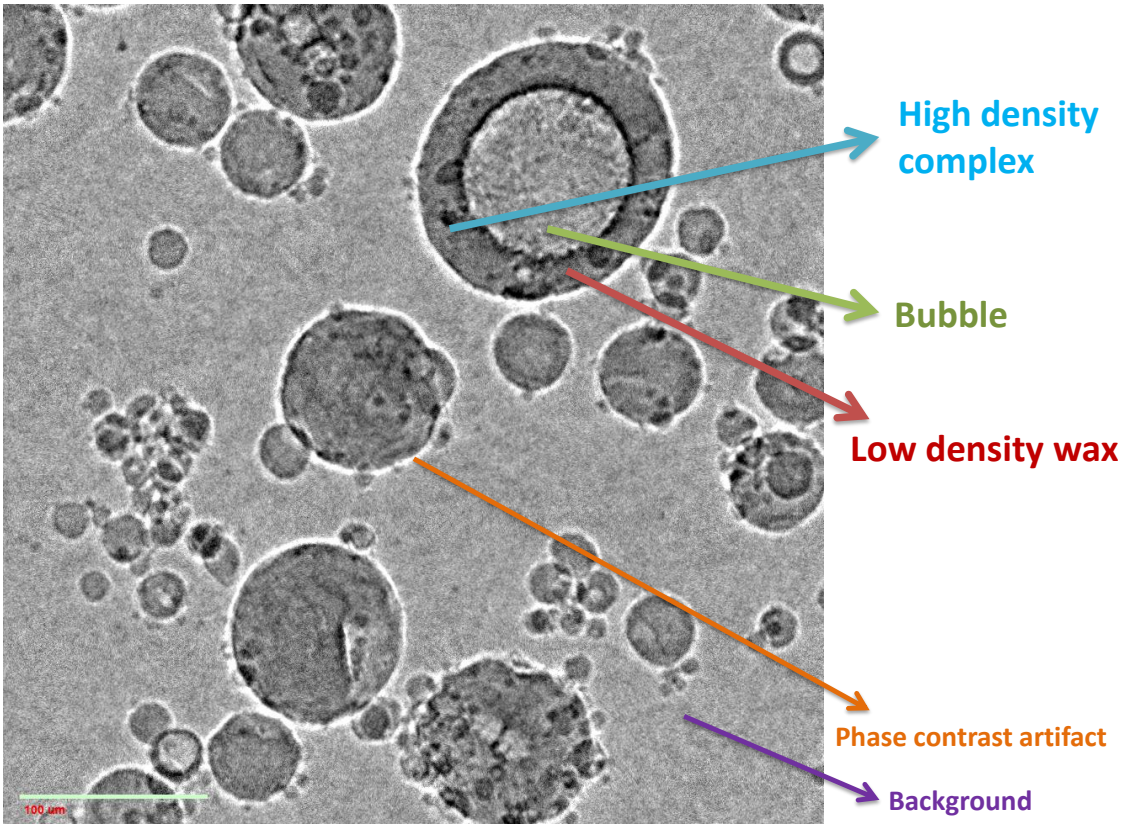

Figure B.2 The X-ray transmission images of the 2APra-$ZnCl_2$ microcapsules with a flavor loading of (0.1185±0.0054) % (mean±SD, n=3).

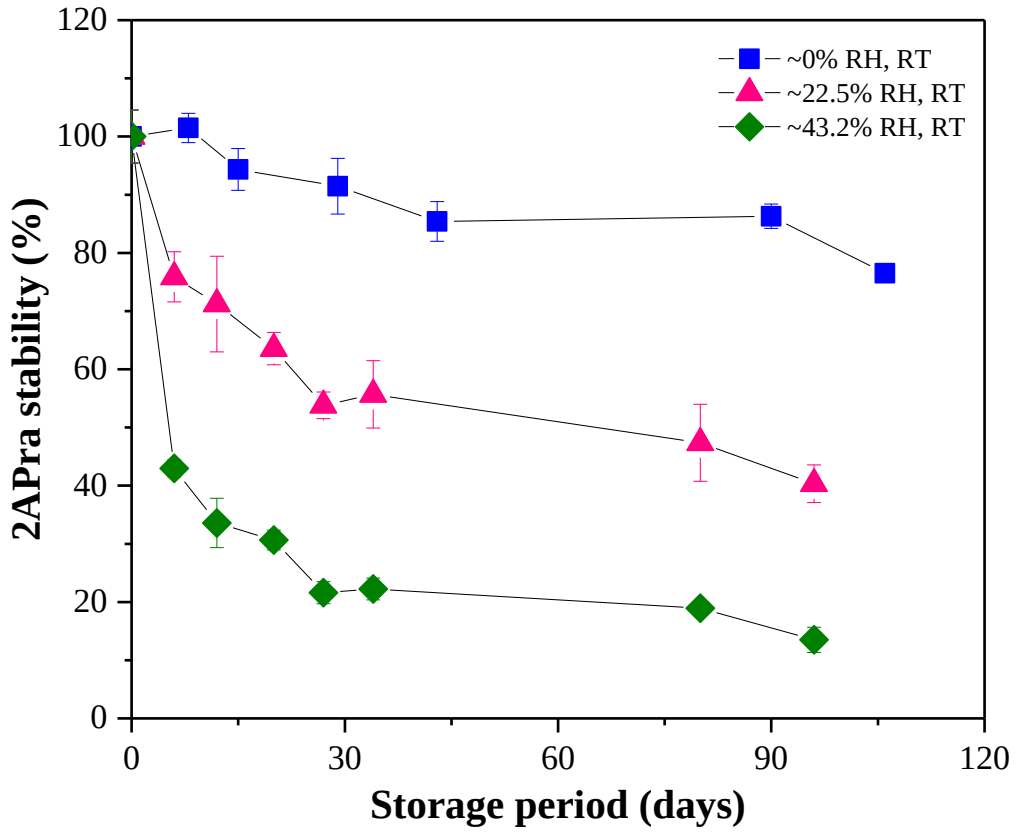

Figure B.3 Flavor stability of 2APra-ZnCl2 microcapsules stored in ~0%, ~22.5% and ~43.2 RHs, respectively (Mean±SD, n=3). Different letters having the same color text indicate significant sample differences as a function of storage period ($p < 0.05$).

APPENDIX C

Comparison of surface morphology characteristics between regular octacosane, spray-chilled octacosane, and spray-chilled complex and octacosane

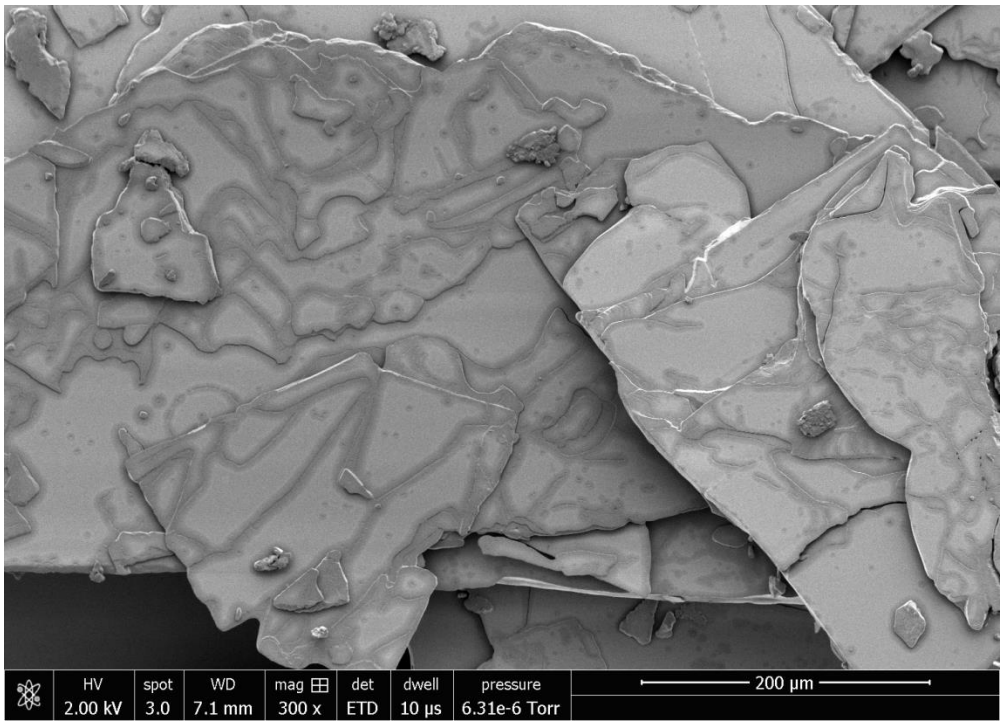

Figure C.1 SEM graph of regular octacosane at 300×magnification.

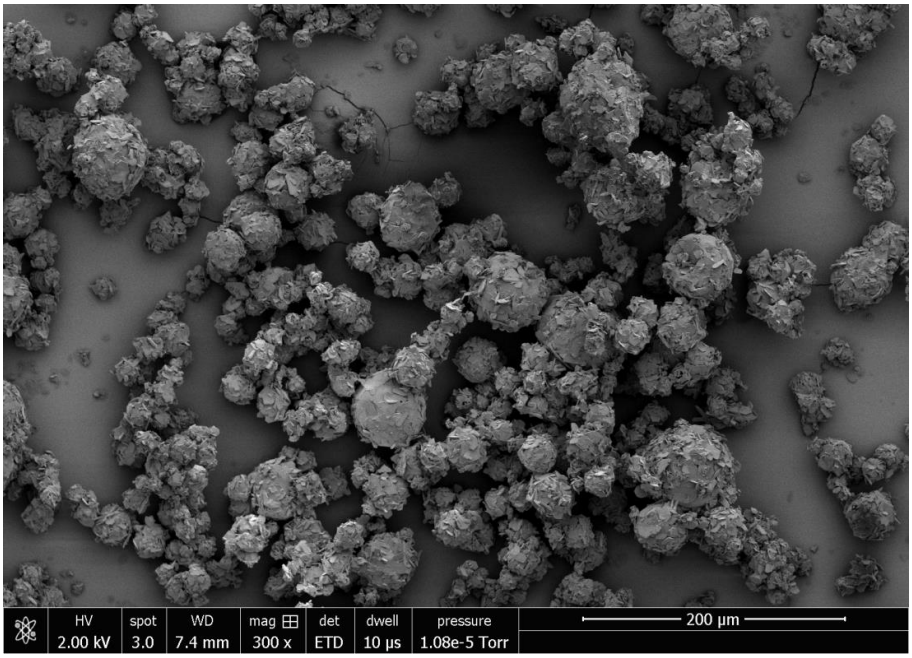

Figure C.2 SEM graph of spray-chilled pure octacosane particles at 300×magnification.

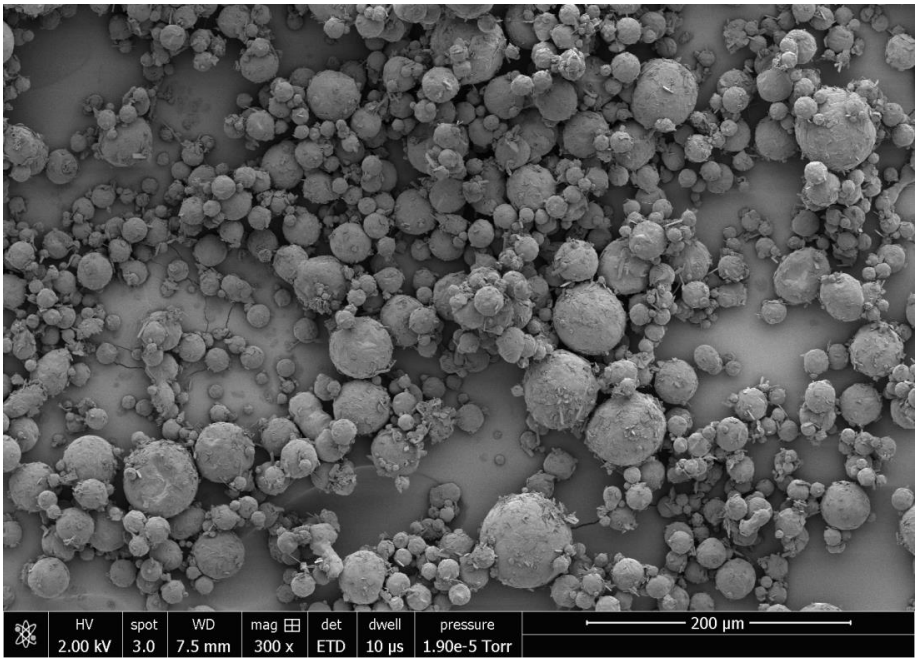

Figure C.3 SEM graph of spray-chilled 2AP-$ZnCl_2$ octacosane microcapsules at 300×magnification.

APPENDIX D

Standard curve for measuring zinc chloride ($ZnCl_2$) content

Zinc dust	110.7	mg		diluted to 1 L			
Concentration of Zinc solution	0.1107	mg/ml, ug/ul					
	Zinc stock solution (ul)	Zinc content (ug)	ZnCl2 content (ug)	OD_{620}	DI water (ul)	Zincon dye (ul)	Borate buffer (ml)
Point 1	0	0	0	0.0247	70	100	3
Point 2	5	0.5535	1.15402803	0.0845	65	100	3
Point 3	10	1.107	2.30805606	0.1424	60	100	3
Point 4	15	1.6605	3.46208409	0.1993	55	100	3
Point 5	20	2.214	4.61611211	0.2698	50	100	3
Point 6	25	2.7675	5.77014014	0.3028	45	100	3

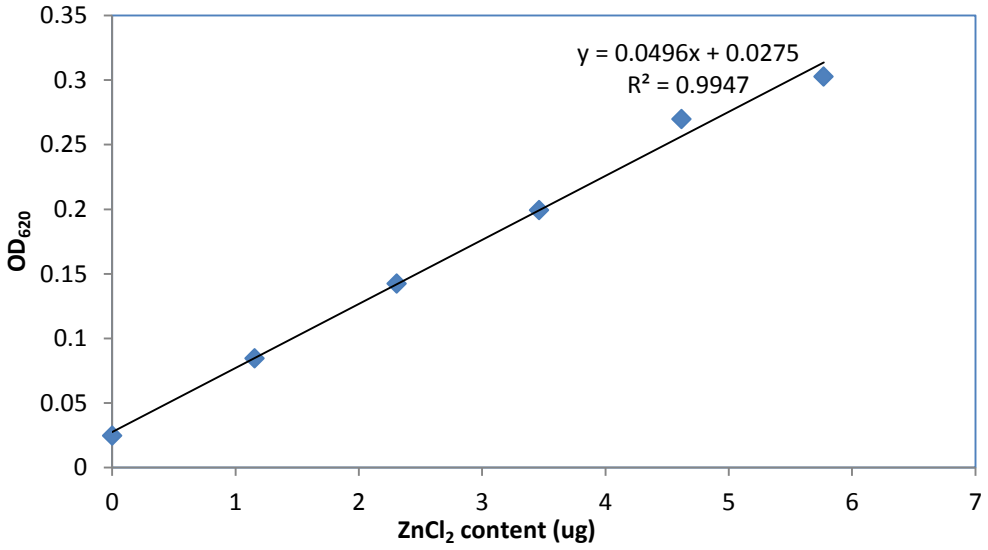

Figure D.1 Standard curve for measuring zinc chloride ($ZnCl_2$) content.

APPENDIX E

Response factor of 2-acetylpyrazine (2APra)

Internal standard	2-Acetylpyrridine (2APri)	517	µg/µl							
Target compound	2-Acetylpyrazine (2APra)	1.760	mg/ml, µg/µl							

	Conc of 2APra (µg/µl)	Added 2APra (µl)	Mass of 2APra (µg)	Conc of I.S. (µg/µl)	Added I.S. (µl)	Mass of I.S. (µg)	Mass ratio (2APra:I.S.)	Area of 2APra	Area of I.S.	Area ratio (2APra:I.S.)
1	1.760	500	880	0.517	500	258.5	3.4043	19816854	6835246	2.8992
2	0.880	500	440	0.517	500	258.5	1.7021	9708009	6604261	1.4700
3	0.440	500	220	0.517	500	258.5	0.8511	4530750	6567275	0.6899
4	0.220	500	110	0.517	500	258.5	0.4255	2390596	7604805	0.3144
5	0.110	500	55	0.517	500	258.5	0.2128	654558	4658688	0.1405
Slope=	**0.8683**									
R_f=	**1.1517**									

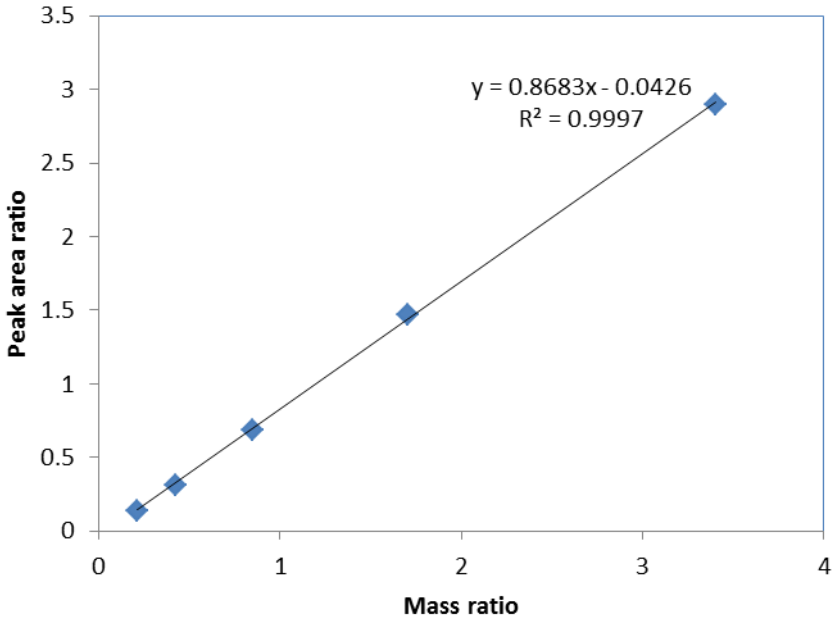

Figure E.1 Response factor of 2-acetylpyrazine (2APra).

APPENDIX F

General protocol of flavor loading determination for spray-chilled microcapsules made with candelilla wax

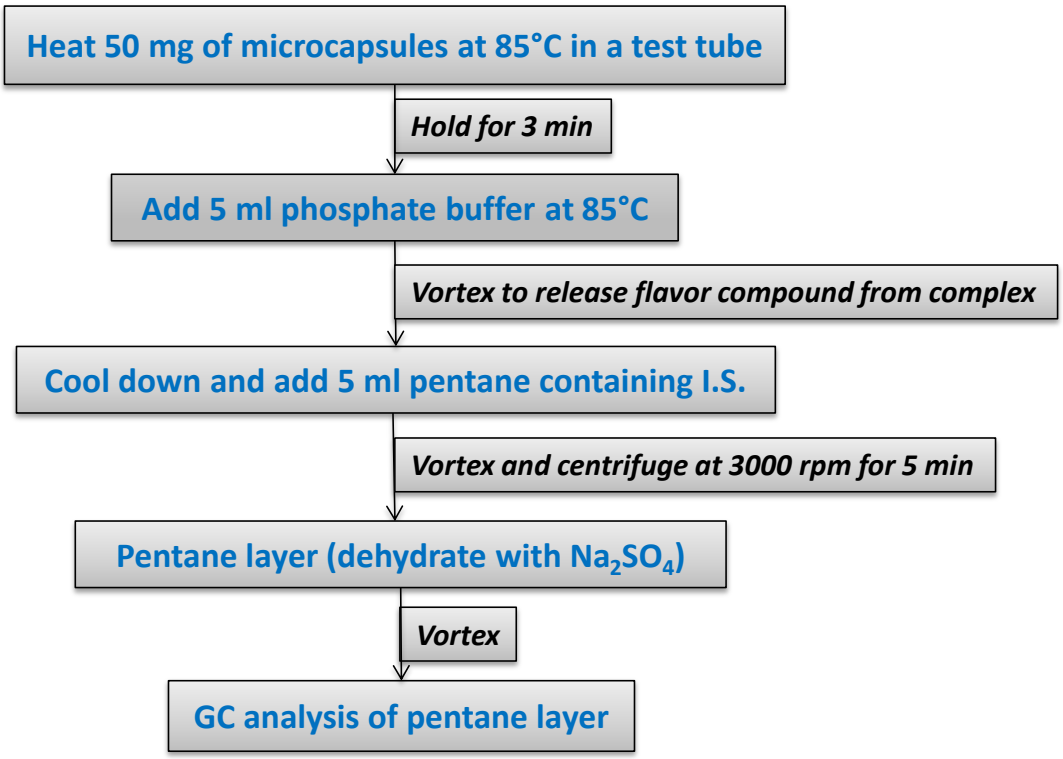

Figure F.1 General protocol of flavor loading determination for spray-chilled microcapsules made with candelilla wax.