FEASIBILITY OF SPRAY CHILLING ENCAPSULATION FOR PROTECTION AND STABILIZATION OF FLAVOR COMPOUND-ZINC ION COMPLEXES

BY

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THESIS

Submitted in the partial fulfillment of the requirements for the degree of Master of Science in Food Science and Human Nutrition with a concentration in Food Science in the Graduate College of the University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

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ABSTRACT

The aim of this study was to investigate the feasibility of utilizing spray chilling encapsulation technology to protect the flavor compound-zinc ion complex, 2-acetylpyridine-ZnCl\textsubscript{2} (2-APri-ZnCl\textsubscript{2}). 2-APri-ZnCl\textsubscript{2} was loaded at 5% (w/w) in molten Hydro-KoteC\textsuperscript{®}, a commercial vegetable stearin. Spray chilling of this suspension was performed using a laboratory scale Mini Spray-dryer B-290 equipped with a spray chilling accessory, a dehumidifier and a two-fluid nozzle (nozzle cap, $\varnothing=2.2$ mm; nozzle tip, $\varnothing=1.4$mm; 046376). The heating temperature ($94^\circ$C) was set at 40% above the melting point of the lipid ($60$-$63^\circ$C). Two independent trials were performed.

Microencapsules were stored in 20mL sealed amber vials and stored at ambient temperature ($25^\circ$C). The percent change in 2-APri-ZnCl\textsubscript{2}, was determined using gas chromatography-flame ionization detection (GC-FID) analysis at 0, 14, 21, 30, and 70 days. Scanning (SEM) and transmission electron microscopy (TEM) were used for micro-level observation of the physical structure of the microencapsules. SEM images of samples from the lower chamber and sample vessel compartments of the spray chiller showed that the materials had physical differences related to flowability (e.g., rough vs. smooth walls) as well as an inhomogeneity with respect to particle size. Samples from the lower chamber exhibited the preferred morphological characteristics in that they had smooth walls and were of relatively homogenous size. Stability studies showed that the microencapsulated 2APri-ZnCl\textsubscript{2} complex with 2.5% initial loading in trials 1 and 2 maintained approximately 80 and 59% retention of
2APri, respectively, after 70 days of storage at ambient temperature. The difference in stability between the two trials is thought to be due to a variance in the operational parameters of the spray chiller.

Results indicate that spray-chilling encapsulation is a viable technique for the protection of the flavor compound-zinc ion complex 2-APri-ZnCl₂, and may be applicable for the protection of the much less stable complexes, 2-acetyl-1-pyrroline-ZnI₂ and 2-acetyl-1-pyrroline-ZnCl₂. Spray-chilling encapsulated flavor compound-zinc ion complexes may be useful as flavoring materials in industrial food applications (e.g., bakery, ice cream, and soups).
To God I dedicate this degree.

And to my grandfather, pillar to our family, who I miss every day.
ACKNOWLEDGEMENTS

To my advisor Dr. Keith Cadwallader without whom this would have not been possible, I am forever thankful for his guidance and support as well as his encouragement through my master’s studies and for allowing me the privilege of being his student. I would also like to thank my committee Dr. Graciela Padua and Dr. Youngsoo Lee for their help, time, effort and willingness to serve on my committee. Also to my life friends for their words of encouragement, Adalberto, Patty, Alex, Janeth, Evelin, Lyda, Ana, Heike and Mauro, Zamorano professors Dr. Gernart, Dr. Matamoros and Dr. Voegtlin for all their help. My friends Monica, Nils, Nadine, Ber, Gizem, Ginn and Gulcin as well as labmates, who with their help and teaching have made this journey of studies lighter, especially Dr. Ming-Chih Fang, John Jerrell, Edibe, Liz and both Bethanys. Finally and foremost, I would extend my gratitude and respect to my daughter Andrea and son Eduardo, the two most important people in my life; and to my family’s unconditional support, special thanks to my mother, brother and sister in law (Lem and Mary), grandmother and father.
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2-Acetyl-1-pyrroline (2AP) has been reported to form both biologically and through Maillard reactions in food materials. Buttery et al. (1982) was first to identify this potent aroma compound. It has a very low odor threshold of 0.1 μg/Kg in water and it is described as a pleasant nutty, rice-like, popcorn-like aroma. It has since been reported as an important odorant in numerous foods (Buttery et al., 1983; Schieberle et al., 1987; Hofmann et al., 1998). Extensive studies have been done in different science disciplines (Plant breeding-Biotechnology, Food Science and Chemistry) to enhance the agronomic and genetic factors for 2AP formation in plants, to achieve its practical chemical synthesis and to find ways to stabilize the compound and enable its potential use as a flavoring material. Only a small number of studies have examined ways to protect or stabilize 2AP, including coating, encapsulation and complexation technologies.

Coating techniques have been strategically used in efforts to stabilize 2AP contained in biological materials or extracts. Laohakunjit and Kerdchoechuen (2007) used as a source of 2AP a pandan (Pandanus amaryllifolius Roxb.) leaf extract produced by supercritical fluid extraction with carbon dioxide. They attempted to stabilize the 25% leaf extract using a 30% sorbitol-plasticized rice starch film. They further demonstrated the potential application of this coating technology to impart 2AP aroma to a nonaromatic rice. Tulyathan et al. (2008) used a 3% brown rice flour gel coating in an effort to stabilize the 2AP content of aromatic rice during storage.
Neither of the above approaches was able to stabilize 2AP to any reasonable extent and additional packaging was necessary to provide better protection.

Studies have been concerned with increasing the stability of 2AP by encapsulation or complexation technology. These are limited to the use of spray-drying technology, vacuum self-drying and ß-cyclodextrin inclusion complexation of either biological extracts or chemical synthesized 2AP.

Apintanapong et al. (2003) used spray-drying to encapsulate 2AP isolated from pandan leaves using different ratios of gum acacia and maltodextrin as carrier materials. They reported that the encapsulated 2AP (0.003% concentration) maintained about 70% retention after 72 days of storage at room temperature. However, the very low overall loading of 2AP makes this approach impractical.

A patent (Srinivas et al., 2006) claims an improved process to stabilize 2AP (chemically synthesized) by vacuum shelf-drying or spray-drying with various binding materials (gum acacia or/and starch), emulsifiers (Tween 80 or 60) and two 2AP:binder ratios (1:20000 or 1:2000). However, the study did not provide any retention and storage stability data. Products made using the encapsulated flavor were considered acceptable based on sensory tests.

In another patent, Andreas et al. (2012) applied spray-drying technology using maltodextrin/ß-cyclodextrin to encapsulate a pandan leaf extract as the source of 2AP. Although the patent demonstrates potential application, no storage or stability studies were mentioned, thus making it difficult to gauge its feasibility.
Recently, a state of the art method has been proposed (Fang, 2013) to stabilize 2-acetyl-1-pyrroline by complexation with zinc ions (ZnI₂, ZnBr₂ or ZnCl₂). Storage stability studies demonstrated that the 2AP-zinc iodide complex was stable (greater than 94% retention) at room temperature (25°C) for up to 3 months. However, a double protection is needed to diminish the anticipated detrimental effects of environmental factors such as moisture. Because of the inherent instability of 2AP and the hydrophilic characteristics of the 2-AP-zinc ion complex (Fang, 2013) a hydrophobic barrier is preferred to further enhance stability and the extend shelf-life.

In order to understand the behavior of flavor compound-zinc ion complex in a hydrophobic wall encapsulate a more stable compound is preferred. Therefore, 2-acetylpyridine-ZnCl₂ (2-APri-ZnCl₂) was selected to serve as the surrogate/core for this model. The objectives of this research were to : 1) determine the feasibility of using spray chilling to protect a flavor compound-zinc ion complex, specifically the stable 2-acetylpyridine-ZnCl₂, 2) determine stability of the microencapsulate stored at ambient temperature for 70 days, 3) determine morphological characteristics of the microencapsulate, and 4) provide recommendations for applying the technique to less stable zinc ion complexes such as 2-acetyl-1-pyrroline-ZnCl₂/ZnI₂ and 6-acetyltetrahydropyridine-ZnCl₂/ZnI₂ to further enhance their stability so these unstable compounds can be used as food flavorings.
1.1 References


2.1 2-Acetyl-1-pyrroline formation and occurrence in foods.

The potent aroma compound 2-acetyl-1-pyrroline (2AP) may be formed in foods by biological means or chemically via the Maillard reaction. Two main biologically sources have been well studied, including aromatic rice (*Oryza sativa*, L) and species belonging to *Pandanus* (Pandanaceae family) (Wongpornchai et al., 2003; Thimmaraju et al., 2005; Yoshihashi et al., 2005; Routry & Rayaguru, 2010). Formation of 2AP through Maillard reaction pathways may involve either ornithine or proline. The amino acids are also involved in the formation of 6-acetyltetrahydropyridine(s) (ATHP) (Hofmann et al., 1988). It is well documented that 2AP is present in a myriad of foods (Schieberle, 1991; Buttery et al., 1994; Karagul-Yuceer et al., 2001; Lee et al., 2001) and has been identified as a key aroma component of aromatic rice (Buttery and Ling, 1982; Buttery et al., 1986). In fact positive sensory attributes of aromatic rice have been directly correlated to the content of 2AP (Tulyathan et al., 2008).

**Biological sources:**

*Pandanus* (Pandanaceae family) contains approximately 600–700 species and plants that are palm-like trees, shrubs and root climbers, with size ranging from small shrubs to medium size trees (<1m-20m). They are native to the “old world” and are dispersed from Africa to the Pacific islands (mainly in Madagascar and Malaysia).
(Routry & Rayaguru, 2010). They are currently distributed along southern India, Southeast Asia, Indonesia and western New Guinea.

*Pandanus odoratissimus*, L. (flowers are scented) and *Pandanus amaryllifolius* Roxb. (aromatic leaves) possess special aromas which are of great interest to the flavor industry as a natural source of 2AP. *Pandanus amaryllifolius* is widely cultivated in Thailand, Malaysia, Indonesia and India. The aroma of the leaves is very distinctive, described as a pleasant and nutty. For this reason, pandan leaves have been widely used as a traditional means for flavoring of food items in countries such as India and Thailand.

Another biological source of 2AP is aromatic rice (*Oryza sativa* L.). Rice is considered a staple food in Asia, Latin America and some parts of Africa. According to World Rice Statistics and FAOSTAT rice provides 20% of the daily calories for more than 3.5 billion people. (World Rice Statistics, [http://www.irri.org](http://www.irri.org)). Basmati and Jasmine are the preferred aromatic rice varieties in the world market and are considered of distinctive high quality and high-valued over non-aromatic rice varieties. Traditional aromatic rice producers included Thailand, India, and Pakistan. Aromatic rice demand has grown in exotic markets such as Europe, Middle East, United State of America, Hong Kong, Ivory Coast. However, its demand has not yet been satisfied. Tables 2.1 and 2.2 illustrate the growing demand/exports of aromatic rice. The largest rice producers are in Asia, including China, India, Indonesia, Bangladesh, Viet Nam, Myanmar, and Thailand. Brazil is the largest producer outside of Asia. Although China and India are the two largest producers, Thailand and Vietnam are the two largest rice exporters (FAOSTAT 2012).
Table 2.1 Jasmine rice export from Thailand 2007-2011

<table>
<thead>
<tr>
<th>Country</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>Average</th>
<th>Export Share (%)</th>
<th>Growth Rate 07-11 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The U.S.</td>
<td>200.27</td>
<td>301.38</td>
<td>340.77</td>
<td>406.48</td>
<td>451.73</td>
<td>340.12</td>
<td>22.70</td>
<td>125.56</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>125.31</td>
<td>149.31</td>
<td>166.32</td>
<td>166.02</td>
<td>165.01</td>
<td>154.39</td>
<td>10.30</td>
<td>31.68</td>
</tr>
<tr>
<td>China</td>
<td>132.80</td>
<td>119.50</td>
<td>108.58</td>
<td>126.47</td>
<td>110.13</td>
<td>119.50</td>
<td>7.98</td>
<td>-17.07</td>
</tr>
<tr>
<td>Singapore</td>
<td>70.97</td>
<td>91.95</td>
<td>100.86</td>
<td>103.41</td>
<td>106.87</td>
<td>94.81</td>
<td>6.33</td>
<td>50.58</td>
</tr>
<tr>
<td>Côte d'Ivoire</td>
<td>64.54</td>
<td>57.61</td>
<td>141.70</td>
<td>116.25</td>
<td>84.83</td>
<td>92.99</td>
<td>6.21</td>
<td>31.43</td>
</tr>
<tr>
<td>Gana</td>
<td>46.43</td>
<td>79.63</td>
<td>67.42</td>
<td>83.01</td>
<td>124.52</td>
<td>80.20</td>
<td>5.35</td>
<td>168.19</td>
</tr>
<tr>
<td>Canada</td>
<td>40.11</td>
<td>66.21</td>
<td>70.46</td>
<td>83.59</td>
<td>82.36</td>
<td>68.55</td>
<td>4.58</td>
<td>105.34</td>
</tr>
<tr>
<td>Malaysia</td>
<td>58.13</td>
<td>80.12</td>
<td>88.86</td>
<td>53.99</td>
<td>36.34</td>
<td>63.49</td>
<td>4.24</td>
<td>-37.48</td>
</tr>
<tr>
<td>Australia</td>
<td>31.29</td>
<td>58.23</td>
<td>65.33</td>
<td>69.72</td>
<td>66.92</td>
<td>58.30</td>
<td>3.89</td>
<td>113.87</td>
</tr>
<tr>
<td>France</td>
<td>24.53</td>
<td>37.65</td>
<td>42.17</td>
<td>43.43</td>
<td>41.11</td>
<td>37.78</td>
<td>2.52</td>
<td>67.59</td>
</tr>
<tr>
<td>Others</td>
<td>290.93</td>
<td>375.94</td>
<td>418.44</td>
<td>434.20</td>
<td>421.15</td>
<td>388.13</td>
<td>25.91</td>
<td>44.76</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1,085</td>
<td>1,417</td>
<td>1,610</td>
<td>1,686</td>
<td>1,690</td>
<td>1,498</td>
<td>100</td>
<td>55.80</td>
</tr>
</tbody>
</table>

Source: ESAAN Center for Business and Economics Research, 2012
Table 2.2 Basmati rice export from India 2010-2013

<table>
<thead>
<tr>
<th>Product: Basmati Rice</th>
<th>2010-11</th>
<th>2011-12</th>
<th>2012-13</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Country</strong></td>
<td><strong>Qty</strong></td>
<td><strong>Value</strong></td>
<td><strong>Qty</strong></td>
</tr>
<tr>
<td>Iran</td>
<td>4,50,657.16</td>
<td>446.24</td>
<td>6,14,922.16</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>6,22,704.06</td>
<td>687.35</td>
<td>7,21,245.48</td>
</tr>
<tr>
<td>United Arab Emirates</td>
<td>6,34,769.33</td>
<td>623.03</td>
<td>7,28,823.29</td>
</tr>
<tr>
<td>Iraq</td>
<td>36,907.90</td>
<td>36.27</td>
<td>1,51,961.25</td>
</tr>
<tr>
<td>Kuwait</td>
<td>1,97,590.40</td>
<td>239.5</td>
<td>1,99,869.77</td>
</tr>
<tr>
<td>Yemen Republic</td>
<td>70,042.10</td>
<td>65.11</td>
<td>92,112.14</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>77,384.45</td>
<td>77.18</td>
<td>1,41,666.21</td>
</tr>
<tr>
<td>United States</td>
<td>47,489.73</td>
<td>54.92</td>
<td>91,816.94</td>
</tr>
<tr>
<td>Jordan</td>
<td>25,602.15</td>
<td>24.96</td>
<td>52,928.98</td>
</tr>
<tr>
<td>Qatar</td>
<td>7,667.74</td>
<td>10.03</td>
<td>16,012.28</td>
</tr>
<tr>
<td>Netherlands</td>
<td>23,097.67</td>
<td>23.45</td>
<td>37,275.22</td>
</tr>
<tr>
<td>Belgium</td>
<td>9,207.06</td>
<td>9.85</td>
<td>23,279.84</td>
</tr>
<tr>
<td>Oman</td>
<td>6,169.88</td>
<td>7.98</td>
<td>18,292.27</td>
</tr>
<tr>
<td>Italy</td>
<td>12,983.74</td>
<td>13.32</td>
<td>24,044.45</td>
</tr>
<tr>
<td>Canada</td>
<td>16,531.47</td>
<td>22.06</td>
<td>28,456.45</td>
</tr>
<tr>
<td>Mauritius</td>
<td>14,180.28</td>
<td>16.02</td>
<td>23,304.46</td>
</tr>
<tr>
<td>Australia</td>
<td>8,715.03</td>
<td>12.13</td>
<td>15,860.04</td>
</tr>
<tr>
<td>Bahrain</td>
<td>5,371.46</td>
<td>7.15</td>
<td>13,847.62</td>
</tr>
<tr>
<td>Georgia</td>
<td>4,920.06</td>
<td>5.34</td>
<td>9,714.14</td>
</tr>
<tr>
<td>France</td>
<td>8,544.95</td>
<td>9.09</td>
<td>14,829.13</td>
</tr>
<tr>
<td>Germany</td>
<td>4,820.59</td>
<td>5.95</td>
<td>17,922.34</td>
</tr>
<tr>
<td>Israel</td>
<td>5,496.18</td>
<td>5.45</td>
<td>9,887.97</td>
</tr>
<tr>
<td>South Africa</td>
<td>3,366.80</td>
<td>4.23</td>
<td>8,127.15</td>
</tr>
<tr>
<td>Spain</td>
<td>2,254.00</td>
<td>2.47</td>
<td>8,178.60</td>
</tr>
<tr>
<td>Reunion</td>
<td>2,605.28</td>
<td>2.94</td>
<td>5,177.22</td>
</tr>
<tr>
<td>Others</td>
<td>6,388.21</td>
<td>7.21</td>
<td>5,722.29</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>23,70,658.41</td>
<td>2,491.12</td>
<td>31,78,174.42</td>
</tr>
</tbody>
</table>

Source: DGCIS Annual Export
Rice can be classified into six groups based on their isozymes: *japonica*, *aromatic*, *indica*, *aus*, *rayada*, and *ashina* (Glaszmann, 1987) (Table 2.3). Simple Sequence Repeats (SSRs) proposed that *Oryza sativa* L. could be categorized into five groups (Garris et al., 2004). Data from the above two studies revealed that the aromatic subpopulation, such as Basmati and Jasmine, were genetically related to the *japonica* subpopulations (Table 1). Glaszmann (1987) claimed in his study that most of aromatic rice cultivars belong to group I (Jasmine) V (Basmati). These groups, which are native to Asia, may also be found in Middle Eastern countries. Therefore, the demand of aromatic rice has not yet been met since these varieties have been of exclusive production in its endemic areas and thus aromatic varieties are emerging from different areas such as US because of molecular or plant breeding efforts.

### Table 2.3 Isozyme based distribution of domestic rice in Asia*

<table>
<thead>
<tr>
<th>Origin</th>
<th>Type</th>
<th>Enzymatic groups</th>
<th>Intermediates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I    II   III  IV  V   VI</td>
<td></td>
</tr>
<tr>
<td>Bangladesh</td>
<td>Deepwater</td>
<td>10  1   5    11  -    -   -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T. Aman</td>
<td>28  -    -    -    2    -    -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aus</td>
<td>2    32   -    -    -    -    -</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Boro</td>
<td>2    6    -    -    -    -    -</td>
<td></td>
</tr>
<tr>
<td>Thailand, Laos</td>
<td>Lowland</td>
<td>168  -    -    -    -    -    -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Upland</td>
<td>12   -    -    -    -    -    -</td>
<td>64</td>
</tr>
<tr>
<td>Java, Bali</td>
<td>Tjerch</td>
<td>10   -    -    -    -    -    -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gundil</td>
<td>4    -    -    -    -    -    -</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Bulu</td>
<td>-    -    -    -    -    -    -</td>
<td>24</td>
</tr>
<tr>
<td>China</td>
<td>Hsien</td>
<td>84   -    -    -    -    -    -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Keng</td>
<td>-    -    -    -    -    -    -</td>
<td>26</td>
</tr>
<tr>
<td>Korea, Japan</td>
<td></td>
<td>2    -    -    -    -    -    -</td>
<td>89  2</td>
</tr>
</tbody>
</table>

*Distribution of well-known varietal based on isozyme (Glaszmann, 1987)*
Because of the importance inherent to 2AP (aromatic characteristic of high-valued aromatic rice and its demand, as well as different thermal processed foods) it is of relevance to rice industry and food industry to further stabilize its aromatic characteristic throughout the shelf-life of a food product.

2.2 Stability of 2AP: biological studies, synthesis and encapsulation

Aromatic rice (*Oryza sativa* L.) and *Pandanus* (Pandanaceae family) contain several volatile compounds, however, it is well known that 2AP is the most impactful odorant in aromatic. However, maintaining its stability has been challenging. Consequently several studies have been reported on 2AP. Various approaches have been explored, including plant breeding, coating techniques, chemical synthesis and scarce studies on encapsulation techniques.

2.2.1 Plant breeding and molecular breeding

Molecular breeding (marker-aided selection) and genetic engineering have played an important role to understand the pathway of the biosynthesis of 2AP and have aided in screening of the desired traits. In 2008, a U.S. patent for “*transgenic rice plants with reduced expression of Os2AP and elevated levels of 2-acetyl-1-pyroline*” claimed to discover genes controlling 2AP synthesis in Jasmine rice by use of genetic engineering technology (Napasintuwong, 2012). Consequently, this had aided grain producers in the improvement of rice varieties. Furthermore, it has made it possible to grow aromatic rice varieties in countries outside of traditional growing
regions. The net result has been an increase in the competition among aromatic rice producers.

2.2.2 Coating technique

Edible coatings have strategically been used for extending shelf life in produce and fruits as well as for the enrichment of foods in essential nutrients (e.g., iron, vitamin A) (Peil et al., 1981). Additional effects such as lipid oxidation, discoloration and off-flavor have been found to decrease as a result of the protection from surface coatings during storage (Tulyathan et al., 2004).

A coating technique was explored to impart 2AP aroma to nonaromatic rice (Laohakunjit and Kerdchoechuen, 2007). In this study, a 25% leaf extract of *Pandanus amaryllifolius* Roxb. (produced by supercritical fluid extraction with carbon dioxide) was dispersed in a 30% sorbitol-plasticized rice starch film. Three nonaromatic rice varieties (RD23, SPR1 and SPR90) were coated with this material, with two aromatic rice varieties (KDML and PTT1) serving as controls. The coated and uncoated samples of non-aromatic rice and uncoated aromatic rice samples were subsequently packaged in bags (nylon15/PE20/LLDPE75) and stored at 25°C for six months. GC-MS analysis showed similar flavor volatile profiles for both aromatic varieties and coated non-aromatic rice controls. Furthermore, coated nonaromatic rice contained higher 2AP levels than one aromatic variety PTT1. Storage data also revealed that 2AP levels decreased during storage. While this is an encouraging approach to improve aroma in non-aromatic rice and could potentially reduce oxidative rancidity during storage, further research needs to be done to show its efficacy in stabilizing 2AP.
Another study (Tulyathan et al., 2008) investigated the effect of coating brown rice with flour gel (3%) containing 2AP and hexanal. Samples were vacuum packaged (PP and laminated OPP/Al/LLDPE) and stored under ambient conditions (27–32°C, 54–62% RH) for four weeks prior to analysis of 2AP and n-hexanal contents at intervals of one month for up to six months. Result suggested that coating of brown rice with the flour gel did not preserve 2AP content during storage, and furthermore coated samples showed lower 2AP content compare to non-coated samples. However, results showed that packaging in laminated OPP/Al/LLDPE (57% 2AP content) offered a better protection over PP (41% 2AP content) for preservation of 2AP and hexanal contents.

2.2.3 Encapsulation techniques: studies and patents

There are limited studies concerned with maintaining 2AP stability by means of encapsulation or inclusion complexation technology. According to literature, previous attempts to increase 2AP stability are limited to the use of spray dry (SD) technology, vacuum self-drying and β-cyclodextrin (β-CD) inclusion complexing of either biological extracts containing 2AP or chemically synthesized 2AP.

Apintanapong et al.(2003) used spray drying technology to encapsulate 2AP using different ratios of gum acacia:maltodextin as carrier material. They used pandan (Pandanus amaryllifolius) leaves as the source of 2AP obtained by steam distillation. They reported poor stability of 2AP (63% reduction) in aqueous basic solution after 7 days storage. However, encapsulated 2AP (0.003% concentration) showed 70% retention 72 days of storage.
A patent (Srinivas et al., 2006) claims an improved process to stabilize 2AP by vacuum shelf-drying (30-60°C; 24” vacuum) or spray drying (140°C) of solution containing synthetic 2AP dispersed in gum acacia or starch and an emulsifier (Tween 80 or 60). The ratio of 2AP to binding material was 1:2000. Studies on the use of the patented material demonstrated overall preferences of products containing the encapsulated flavor. Nonetheless, no storage data was provided.

In another patent Andreas et al. (2012), claimed the use of pandan (Pandanus amaryllifolius) leaves as a source of natural extract of 2AP encapsulated by spray drying (inlet temperature 140-170°C and outlet temperature <80°C) in wall material consisting of maltodextrin and β-cyclodextrin. Specification for the resulting pandan powder were that in contains less than 10ppm of proline, 20 to 50ppm of glutamic acid, 0.5 to 3ppm of 2AP, 100 to 500ppm of total free amino acids and 0.5 to 3ppm of 3-methyl-2(5H)-furanone (based on the total weight). Proposed use of the novel powder included enhancing roasted notes, masking off-odors in lipids and oils in foods. Although the patent encompasses a great deal of application, no storage studies were mentioned to better understand the likelihood of its use in flavoring foods.

2.2.4 Synthesis

Various methods have been reported in the literature for the synthesis of 2AP. Buttery et al. (1982) proposed the first synthesis of 2AP from hydrogenation process via rhodium on alumina catalyst and hydrogen. De Kimpe et al. (1993) proposed a large-scale method which involved the oxidation of methylprolinate to 2-(methoxycarbonyl)-1-pyrroline using Grignard reaction. Duby et al. (1993; 1996)
employed carbonyl-protected analogues to improve the yield due to instability of 2AP.

Fuganti et al., (2007) developed a method for the synthesis of 2AP, 6-acetyl-1,2,3,4-tetrahydropyridine and its tautomer 6-ethyl-2,3,4,5-tetrahydropyridine (ATHP) based on regioselective oxidation of the heterocyclic side chain with selenium dioxide.

Recently, Fang, 2013 proposed a state of the art method for the coordination of 2AP, ATHP, and 2-propionyl-1-pyrroline (2PP) to zinc ions (ZnI₂, ZnBr₂ or ZnCl₂) that formed stable complexes in dry powder form. Stability studies demonstrated that 2-AP-ZnI₂ complex was stable at room temperature (25°C), holding 94% retention for 3 months. However, further protection needs to be done in order to increase its stability due to its sensitivity to environmental factors (temperature, moisture) and interaction with food components. An appropriate encapsulation technique might aid in increasing the shelf life and feasibility the use of this technology in production of a flavoring agent. Therefore, this state of the art method may ultimately be a promising technology for use by the flavor industry.

2.3 Flavor encapsulation technologies.

Flavors are categorized as additives and they can either be volatile (perceived in olfactory epithelium) or non-volatiles (perceived through taste buds) compounds. They can be generated by chemical synthesis or natural extraction. Volatile aroma compounds (aromas) are generally relative low molecular weight \( M_w < 250 \text{ Da} \) compounds with polarities ranging from hydrophilic to hydrophobic. Currently, the
food, feed, cosmetic and pharmaceutical industries represent the largest product segment in the food additives market. According to Transparency Market Research in their article “Food Additives Market (Flavors, Sweeteners, Enzymes, Colorants, Emulsifiers, Shelf-Life Stabilizers and Fat Replacers) - Global Industry Analysis, Size, Share, Trends, Growth and Forecast, 2012 – 2018” the additive segment is expected to exceed USD 12 billion by 2018. Furthermore, flavors are exceptionally important because they make a meaningful contribution in consumers’ overall acceptance of final food products. The tendency of flavors decline or degrade through processing and storage have created interests in their protection via encapsulation techniques prior to its addition in food products (Shahidi & Han, 1993).

Encapsulation can be thought as an elite process that aids to preserve flavor compounds. It was first used in the 1950’s (Green & Scheicher, 1955) in carbonless copying paper and was successfully adapted by the pharmaceutical, cosmetic, food and feed industries. It converts and concentrates volatile compounds from a solid, liquid or gaseous state into small solid particles, usually powder (Porizio, 2004). It can further be described as the process where a core material is protected within a continuous coating film which further releases its content at controlled rates over a period of time (Reineccius & Risch, 1988). Encapsulation technique has made numerous food applications doable. Food ingredients such as flavors, lipids, enzymes, microorganisms, artificial sweeteners, essential oils, vitamins, minerals, preservatives, antioxidants, cross-linking agents, leavening agents, masking agents, colorants, and nutrients are nowadays used were in the past they were not technically possible. (Risch, 1995; Reineccius, 1994). Reineccius (1991) acknowledged the use
of flavor encapsulation as a process that affords protection to vulnerable flavor compounds during processing and storage. Encapsulation may decrease the effects of chemical degradation, evaporation, and oxidation. Furthermore, it can facilitate the use of encapsulated ingredients to be used in food products at the desire time or processing step.

In order to optimize the encapsulation ingredient, some consideration should be taken into account beforehand. Comprehension of key factors (core properties, wall materials, interactions between the core, matrix and the environment, stability of the microencapsulated ingredient in storage (refrigerated or ambient), release mechanisms (fracturation, dissolution, pressure, heat and shear), legal and regulatory requirements for addition into foods should be considered. Finally, the cost of the encapsulated ingredient in the final product should ideally be profitable. A series of stages are needed to produce a final microencapsulated product (Figure 2.1).

Fig.2.1 Microencapsulation Developing process (Sanguansri & Augustin, 2010)
2.3.1 Encapsulation technologies

Numerous mature engineering technologies can be used for encapsulation, including chemical or mechanical processes. Figure 2.2 illustrates the general approach used for encapsulation and Table 2.4 describes the characteristics of some of the available encapsulation techniques.

![Schematic Encapsulation of flavor compounds (Madene et al., 2006)](image)

The most common technologies used are spray drying, spray cooling, coacervation, melt extrusion, fluidized bed, molecular inclusion and freeze-drying. However, the choice of encapsulation technique should base on the final intention of use (protect the flavor during storage or control the release of the flavor) and the core ingredient properties.
Table 2.4. Characteristics of flavor encapsulation processes (Madene et al., 2006)

<table>
<thead>
<tr>
<th>Encapsulation method</th>
<th>Particle size (μm)</th>
<th>Max. load (%)</th>
<th>References</th>
<th>Application area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical technique</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simple coacervation</td>
<td>20–200</td>
<td>&lt;60</td>
<td>Richard &amp; Benoît, 2000</td>
<td>Chewing gum, baked foods</td>
</tr>
<tr>
<td>Complex coacervation</td>
<td>5–200</td>
<td>70–90</td>
<td>Richard &amp; Benoît, 2000</td>
<td></td>
</tr>
<tr>
<td>Molecular inclusion</td>
<td>5–50</td>
<td>5–10</td>
<td>Uhlemann et al., 2002</td>
<td>Instant beverages, confectionery, teas</td>
</tr>
<tr>
<td>Mechanical technique</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spray chilling</td>
<td>20–200</td>
<td>10–20</td>
<td>Uhlemann et al., 2002</td>
<td>Prepared dishes, ices</td>
</tr>
<tr>
<td>Extrusion</td>
<td>200–2000</td>
<td>6–20</td>
<td>Uhlemann et al., 2002</td>
<td>Confectionery, instant drinks, extruded snack</td>
</tr>
<tr>
<td>Fluidized bed</td>
<td>&gt;100</td>
<td>60–90</td>
<td>Richard &amp; Benoît, 2000</td>
<td>Prepared dishes, confectionery</td>
</tr>
</tbody>
</table>

**Drying process** involves the evaporation of water to form the encapsulated core material. Common drying techniques are spray drying, fluidized bed, freeze-drying and tray drying. Spray drying is the preferred technology to encapsulate flavors because it has been widely used and cost effective. However, Reineccius (1988) reported that spray drying initial stages could generate a significant loss of volatile compounds.

**Hot melt process**, transition from glassy to rubbery state

Double encapsulate technique is one the few techniques that can deal with a wide array of coating materials and flavor compounds. It can be used for both
hydrophilic and hydrophobic compounds. It has been subsequently used after spray drying.

Spray chilling and spray cooling are very similar to the spray drying encapsulation. It can be thought as a merging of spray drying and hot melt technologies. It is similar to SD process that both involve dispersing the core material into a liquefied coating material and spraying it through heated nozzles into a controlled environment. They differ from spray drying in the temperature of the air that the drying chamber uses, type of energy (evaporation and solidification) and wall materials used in the two techniques. The cool or chilled air in the chamber causes the coating material to solidify around the core and no water is evaporated from the coating material. In spray chilling, the most commonly used coating materials are molten fractionated and hydrogenated vegetable oils with a melting point of 32–42°C, while vegetable oils or other materials with the melting point of 45–122°C are often used in spray cooling (Risch, 1995), waxes, fatty acids, water insoluble monomers and water soluble polymers. They are most often used to encapsulate solid food additives, such as vitamins and minerals, flavors, starter cultures, lipids and enzymes. This encapsulating technology aims to protect the ingredients from pH, enzyme activity, moisture and oxygen as well as improve microparticle flow, morphological characteristic and gives its kinetic release (Fig. 2.3). The spray chilling end products are water insoluble and can release their contents at or around the melting point of the coating material. The controlled release is a desired property and important to food ingredients in many food applications. This property makes this process suitable to retard volatile compound loss (Dziezak, 1988; Graves and Weiss, 1992; Risch, 1995)
due to thermal processing. Some of the food applications are in bakery products, dry soup mixes, and food containing high levels of fat (Dziezak, 1988; Madene et al., 2006).

Fig. 2.3 Mechanism of flavor release. (a) Erosion, gradually dissolves; (b) Diffusion, flavor diffuses (c) Extraction by mechanical force; and (d) Burst, rupture by mechanical or osmotic forces. (Ubbink & Schoonman, 2003).

The spray chill microencapsulates are matrix type and microparticle morphology is a dense, spherical and smooth surface due to the lack of solvent evaporation (Okuro et al., 2013).

To select an appropriate encapsulation technique will depend on the physical characteristic of the flavor and the processing conditions. Fig 2.4 illustrates the use of different encapsulation technologies and feasibility related to the nature of the ingredients, production capacity and release mechanism. Processes of interest that have been used to encapsulate hydrophilic compounds include spray chilling, spray cooling and fluidized bed.
Fig. 2.4 Summary of the characteristics of common microencapsulation technologies (Adapted Gouin, 2004)

2.3.2 Wall materials

Numerous wall materials have been used to encapsulate flavors. The choice is inherent to the core material and the applications it was originally intended. Wall materials are generally selected from an array of natural (proteins, carbohydrates, lipids and waxes), synthetic (co-polymer) or semi-synthetic (methyl cellulose) sources which may be used alone or in combination. The materials chosen as encapsulants are typically film forming, odorless, tasteless and non-hygroscopic. Other additives, such as emulsifiers, plasticizers or defoaming agents may be added to enhance/ensure the desire characteristics of the final product.

Lipids have been extensively used in the pharmaceutical industry mainly for the spray chilling or congealing techniques (Eldem et al., 1991).

2.3.3 Encapsulation of hydrophilic compounds
The available literature suggests that hydrophilic compounds can be encapsulated by specific techniques to protect the core ingredient. Some of the choices include spray chilling, spray cooling and fluidized bed (Madene et al., 2006).

The use of lipids in the production of microparticles has been known for many years in the pharmaceutical industry, as well as the “spray-chilling” or “congealing” technique (Eldem et al., 1991). Currently the spray-cooling technique is receiving considerable attention, since it is a rapid, safe and reproducible physical process, allowing the easy adjustment of the particle size. The solution, suspension or emulsion containing a core material in a molten lipid matrix is atomized into an environment at a temperature below the melting point of the mixture in use (Albertini et al., 2008), generally a cold chamber (cold air or liquid nitrogen).
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3.1 Abstract

The aim of this study was to investigate the feasibility of utilizing spray chilling encapsulation technology to protect the zinc ion-flavor compound complex, 2-acetylpyridine-ZnCl$_2$ (2-APri-ZnCl$_2$). 2-APri-ZnCl$_2$ was loaded at 5% (w/w) in molten Hydro-KoteC®, a commercial vegetable stearin. Spray chilling of this suspension was performed using a laboratory scale Mini Spray-dryer B-290 equipped with a spray chilling accessory, a dehumidifier and a two-fluid nozzle (nozzle cap, 2.2 mm; nozzle tip, $\emptyset =1.4$mm; 046376). The heating temperature (94°C) was set at 40% above the melting point of the lipid (60-63°C). Two independent trials were performed.

Microencapsules were stored in 20mL sealed amber vials and stored at ambient temperature (25°C). The percent change in 2-APri-ZnCl$_2$, was determined using gas chromatography-flame ionization detection (GC-FID) analysis at 0, 14, 21, 30, and 70 days. Scanning (SEM) and transmission electron microscopy (TEM) were used for micro-level observation of the physical structure of the microencapsules. SEM images of samples from the lower chamber and sample vessel compartments of the spray chiller showed that the materials had physical differences related to flowability (e.g., rough vs. smooth walls) as well as an inhomogeneity with respect to particle size. Samples from the lower chamber exhibited the preferred morphological
characteristics. Stability studies showed that the microencapsulated 2APri-ZnCl2 complex with 2.5% initial loading in trials 1 and 2 maintained approximately 80 and 59% retention of 2APri, respectively, after 70 days of storage at ambient temperature. The difference in stability between the two trials is thought to be due to a variance in the operational parameters of the spray chiller.

Results indicate that spray-chilling encapsulation is a viable technique for the protection of the flavor compound-zinc ion complex 2-APri-ZnCl2, and may be applicable for the protection of the much less stable complexes, 2-acetyl-1-pyrroline-ZnI2 and 2-acetyl-1-pyrroline-ZnCl2. Spray-chilling encapsulated flavor compound-zinc ion complexes may be useful as flavoring materials in industrial food applications (e.g., bakery, ice cream, and soups).
3.2 Introduction

2-Acetyl-1-pyrroline (2AP) is a well-known powerful, yet unstable flavor compound that imparts characteristic roasty and popcorn-like aroma to a myriad of foods (aromatic rice such as basmati and jasmine rice, popcorn, tortillas, bread, etc.). Many studies have been conducted on 2AP. These include general flavor chemistry studies with respect to its identification and quantification in various food products. Studies have also focused on the effect of agronomic parameters (yield, biotechnology) on its formation in plants, especially aromatic rice varieties. Efforts to develop a practical synthesis of 2AP have for the most part been unsuccessful, due largely to the inherent instability of the compound. For this reason investigators have attempted to isolate 2AP from various agricultural materials known to contain appreciable levels of the compound. However, the inherent instability of 2AP has been a major challenge, thus prompting other studies on ways to protect the compound using encapsulation and complexation technologies.

Coating techniques have been strategically used in efforts to stabilize 2AP contained in biological materials or extracts. Laohakunjit and Kerdchoechuen (2007) used as a source of 2AP a pandan (Pandanus amaryllifolius Roxb.) leaf extract produced by supercritical fluid extraction with carbon dioxide. They attempted to stabilize the 25% leaf extract using a 30% sorbitol-plasticized rice starch film. They further demonstrated its potential application using the product to impart 2AP aroma to a non-aromatic rice. Tulyathan et al. (2008) used a 3% brown rice flour gel coating in an effort to stabilize the 2AP content of aromatic rice during storage. Neither of the
above approaches was able to stabilize 2AP to any reasonable extent and additional packaging was necessary to provide better protection.

Studies have been concerned with increasing the stability of 2AP by encapsulation or complexation technology. These are limited to the use of spray-drying technology, vacuum self-drying and β-cyclodextrin inclusion complexation of either biological extracts or chemical synthesized 2AP.

Apintanapong et al. (2003) used spray-drying to encapsulate 2AP isolated from pandan leaves using different ratios of gum acacia and maltodextrin as carrier materials. They reported that the encapsulated 2AP (0.003% concentration) maintained about 70% retention after 72 days of storage at room temperature. However, the very low overall loading of 2AP makes this approach impractical.

A patent (Srinivas et al., 2006) claims an improved process to stabilize 2AP (chemically synthesized) by vacuum shelf-drying or spray-drying with various binding materials (gum acacia or/and starch), emulsifiers (Tween 80 or 60) and two 2AP:binder ratios (1:20000 or 1:2000). However, the study did not provide any retention and storage stability data. Products made using the encapsulated flavor were considered acceptable based on sensory tests.

In another patent, Andreas et al. (2012) applied spray-drying technology using maltodextrin/β-cyclodextrin to encapsulate a pandan leaf extract as the source of 2AP. Although the patent demonstrates potential application, no storage or stability studies are mentioned, thus making it difficult to gauge its feasibility.

A state of the art method for the coordination of 2AP to zinc ions (ZnI₂, ZnBr₂ or ZnCl₂) to form crystalline complexes in a stable dry powder form was reported by
Fang (2013). Additional treatment was suggested to protect the crystalline powder by isolating it from environmental factors (temperature, moisture) and food components (Fang, 2013).

In the present study, an encapsulation technique is explored to further protect the novel flavor compound-zinc ion complex developed by Fang (2013). Spray chilling is the chosen encapsulation technique since it provides the needed moisture barrier to protect the flavor compound-zinc ion complex. Due to the great instability of 2AP-zinc ion complexes, a more stable complex comprised of 2-acetylpyridine-zinc chloride (2APri-ZnCl₂) was used in the present study to help demonstrate the feasibility of the technology. Flavor loading, storage stability and morphological characteristics are described.

3.3 Materials and Methods

3.3.1 Chemicals

2-Acetylpyridine (2APri), collidine (2,4,6-trimethylpyridine), zinc chloride solution (1.0 M in diethyl ether) and zinc chloride USP grade were purchased from Sigma-Aldrich (St Louis, MO, USA). Anhydrous diethyl ether and methylene chloride were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

3.3.2 Encapsulating carrier

A commercial vegetable lipid (stearin) was used as encapsulating agent, Hydro-KoteC® (Abitec, Inc., Columbus, Ohio). Hydro-KoteC® is a hydrogenated cottonseed oil with a melting point 62°C. The materials were kindly supplied by Abitec, Inc., Columbus Ohio.
3.3.3 General procedure for formation of 2-APri-ZnCl$_2$ complex

The general procedure described by Fang (2013) was followed with some modifications. Zinc chloride (0.1M, 8mL) in anhydrous diethyl ether was added to 2Apri (2.09g in 10mL ether) in a 50mL glass centrifuge tube containing a stir bar. The solution was stirred vigorously on a stir plate; during addition, a precipitate of the complex was formed. The precipitate was washed three times with anhydrous diethyl ether (10mL), vortexed and centrifuge before removing solvents. Moderate purging with nitrogen gas was done to obtain the fine powdered complex. The complex was stored at room temperature (25°C) in a silanized glass vial (20mL) sealed with a PTFE-lined silicon cap prior to spray chilling encapsulation.

3.3.4 Encapsulation process
3.3.4.1 Sample preparation

A premix was made by placing 14g of Hydro~KoteC® (flakes) into a silanized glass bottle (20mL) followed by heating in a water bath (approximately 94°C) to facilitate melting of the lipid. The 2-APri-ZnCl$_2$ complex was added to molten lipid matrix which was homogenized with a fixed speed vortex mixer (Genie1-Touch mixer) at 3200rpm for 2min. The homogenized solution appeared as a viscous white/cloudy suspension.

This suspension was added to the product feed vessel of the spray-chilling accessory prior to spray chill encapsulation.
3.3.4.2 Spray chilling

Spray chilling of the above suspension was performed using a laboratory scale Mini Spray-dryer B-290 (BÜCHI Labortechnik AG), equipped with a spray chilling accessory, 230V (040351), a dehumidifier (B-296) and a two-fluid nozzle (nozzle cap, \( \varnothing = 2.2 \text{mm} \); nozzle tip, \( \varnothing = 1.4 \text{mm}; \) 046376). The heating temperature was set at 40% above the melting point of the lipid (e.g. melting point of Hydro-KoteC\(^\circledR\) is between 60°C and 63°C: resulting in heating bath temperature of 1.4 \( \times \) 63°C = 94°C).

The spray chiller was operated according to manufacturer recommendations with atomization airflow set to 30mm rotameter (about 440 liter/h) using compressed air at 80lb/in\(^2\)bar pressure. The system required approximately 1 hour to reach steady state conditions. The dehumidifier (cold trap at 5°C) was used to control relative humidity (17%) of the inlet air, which made the drying conditions more reproducible.

The premix solution (2-APri-ZnCl\(_2\) complex/molten lipid matrix) was slowly added to 36g of melted lipid matrix contained in the product feed vessel of the spray-chilling accessory. The mixture was gently stirred to homogenize before the solution was fed by means of a needle valve to start the spray-chilling encapsulation process. Droplets were dispersed and solidified into powdered like particles (Figure 1). Two trials were performed to test the reproducibility of parameters and the spray chilling encapsulation process.

3.3.4.3 Encapsulation powder collection

Three different sites in the spray chiller apparatus contained encapsulated powder: upper chamber (UC), lower chamber (LC) and sample vessel (SV) (Scheme
3.1). The three sites were collected separately. The encapsulated powders were further weighed to determine the yields, particle size and morphological characteristics. Loading content and stability were determined for powders obtained from the lower chamber and sample chamber.

3.3.4.4 Stability of 2-Apri-ZnCl₂ complex in encapsulated form

The encapsulated powders collected from LC and SV were stored in separate glass amber bottles (125mL) and sealed (airtight) with Teflon-lined closures. The bottles were stored at 25°C for the future assays of stability and imaging (morphology). The stability of encapsulated 2-APri-ZnCl₂ complex was monitored for 0, 14, 21, 30 and 70 d. For analysis, bottles were opened for only a short period of time to allow for sampling of product.

Scheme 3.1. Spray Chilling of 2-APri-ZnCl₂ complex: (a) Pre-mix and (b) spray chilling encapsulation process.
3.3.5 Quantitative Analysis

3.3.5.1 Quantitation of 2-acetylpyridine in 2-Apri-ZnCl₂ complex and in encapsulates

Analyses were carried out as described by Fang (2013) with some modifications. Aqueous phosphate buffer (0.5mL, 50mM, pH=7) was pipetted into a 2mL vial containing a sample of 2-Apri-ZnCl₂ complex (5-10mg). The resulting suspension was extracted with 0.5mL of anhydrous diethyl ether containing collidine as internal standard (7.50mg/mL). The mixture was vortexed for 1min and then centrifuged (3000xg, 5min). The solvent layer was used for GC analysis. The GC response factor (Rf₁) for 2-acetylpyridine against the internal standard (collidine) was determined by following the extraction conditions described above using five levels of 2-acetylpyridine. A typical calibration curve is shown in Appendix A.

All samples were analyzed in duplicate.

Loading of 2-acetylpyridine in 2-APri-ZnCl₂ complex was determined using the following formula:

\[
\text{Loading\%} = \left( \frac{\text{area of 2Arpi}}{\text{area of IS}} \right) \times \left( \text{[conc collidine, mg/mL]} \times 0.5\text{mL} \times \text{Rf1} \right) \\
\div \text{sample weight} \times 100\%
\]

3.3.5.2 Loading analysis of encapsulated 2-APri-ZnCl₂ complex

Samples of encapsulated 2-APri-ZnCl₂ complex (15-20mg) were drawn randomly from glass amber bottles stored at 25°C and weighed into a 1.5mL vial. Aqueous phosphate buffer (0.5 mL, 50 mM, pH = 7) was added to the sample, followed by extraction with 0.5 mL of anhydrous diethyl ether containing collidine as internal standard (7.5mg/mL). The mixture was vortexed for 5min and centrifuged (3000xg,
5 min), and then these steps were repeated in order to better separate the ether phase from the matrix (Scheme 3.2). The GC response factor ($R_f$) for 2-acetylpyridine against the internal standard (collidine) was determined by following the extraction conditions described above using five levels of 2-acetylpyridine in vials containing 15-20 mg of Hydro Kote C. A typical calibration curve is shown in Appendix A.

Scheme 3.2. Encapsulation separation and extraction process before analysis by GC-FID.

Loading of 2-acetylpyridine in 2-AP-ri-ZnCl$_2$ complex encapsulates were determined using the following formula:

$$\text{Loading\%} = \left( \frac{\text{area of 2 Acryl}}{\text{area of IS}} \right) \times ([\text{conc collidine, mg/mL}] \times 0.5 \text{mL} \times R_f2)$$

$$\div \text{sample weight} \times 100\%$$
3.3.6 Gas Chromatography

Gas chromatography was performed using a 5890 series II GC equipped with a split/splitless injector, flame ionization detector (FID) and HP-1 column (30m x 0.32mm x 0.25µm film thickness; Agilent, Palo Alto, CA). Helium was the carrier gas at a constant flow of 1mL/min. The injector was held at 250 °C in the split mode. GC oven temperature was programmed from 50 to 225°C at 10°C/min with initial and final hold times of 5min.

3.3.7 Characterization of encapsulates

Morphological characterization of the encapsulated particles was carried out using both Scanning (SEM) and transmission electron microscopy (TEM).

3.3.7.1 SEM

Scanning electron microscopy was performed using an environmental scanning electron microscope with a field-emission electron gun (XL30 ESEM-FEG; Philips/FEI Co., Hillsboro OR). The instrument may be operated in either normal high-vacuum SEM mode or 'wet' ESEM mode; for this study it was operated in high-vacuum SEM mode at 5 kV, spot 3 (2.1nm). A 25mm diameter carbon double-stick tab was attached to a 25mm diameter aluminum SEM stub, and a small pyramid of dried encapsulated particles was placed in its center. The stub was then tapped gently to distribute the particles evenly across its surface. To aid conductivity, the particles
were then pressed gently into the carbon tab using nonstick release paper. The stub was then coated with ca. 7 nm of gold−palladium using a Denton Vacuum (Mooresstown NJ) Desk-2 turbo sputter coater. Images were collected at various magnifications (See Figures 1, 3 and 4) and exhibited a size range from 7.5µm to 72.2µm in particle size.

3.3.7.2 TEM

Transmission electron microscopy (CM200; Philips/FEI Co.) was performed at 120kV with a spot size of 3 (105nm; indicating the diameter of the electron beam, not the resolution of the microscope). Carbon-stabilized Formvar-coated 400-mesh copper grids (cat. no. 01811; Ted Pella Inc., Redding CA) were glow-discharged for 4 minutes at just under 0.3A in an argon atmosphere using a Denton Vacuum DPG-1 glow-discharge instrument. They were then inverted onto ca. 8 microliters of an aqueous suspension of particles, with an incubation time of 1.5 to 3 minutes. Following three quick rinses in droplets of deionized water, and two quick rinses in 1% aqueous uranyl acetate (the grid was blotted with filter paper, from the side between each rinse), the grid was incubated on a droplet of 1% uranyl acetate for 2 minutes. It was then blotted from the side on filter paper and allowed to dry before being inserted into the TEM and observed. Negative-stained particles could be seen in a range of approximately 15 to 20nm in diameter; a second set of larger particles, in the 120 to 500nm range, was also observed.

3.4 Results and Discussion

3.4.1. Microencapsulate production
3.4.1.1 Morphological characterization of microparticles

Free-flowing powdered microencapsulates were collected from three different sites in the spray chiller apparatus: upper chamber (UC), lower chamber (LC) and sample vessel (SV). LC and SV microencapsulates were analyzed for morphological characteristics using SEM. Micrographs (Fig.1) revealed that the microencapsulated particles exhibited spherical shapes, which is in agreement with previous studies on the spray chilling technique (Savolainen et al., 2002; Chambi et al., 2008; Pedroso et al., 2012). Nonetheless, additional characteristics need to be considered to assess the flow properties. The spherical shape formed in spray chilling encapsulation is a usual characteristic of the technique (Okuro et al., 2013). The LC sample showed a smoother structure, without noticeable fissures at the surface, than the SV sample.

The particle size is an important characteristic in food applications (Madene et al., 2006). A particle size ranging from 20-200µm is preferred; while greater particle size might be a disadvantage in food applications. The results of encapsulates taken from two sites (LC = 9.04µm to 62.7µm; SV = 7.5µm to 72.2µm) indicated that these are within the preferred particle size range.
Fig. 3.1 SEM micrographs of microencapsulates containing 2-APri-ZnCl$_2$ complex A1, A2) LC sample and B1, B2) SV sample.

In this experiment, through two imaging trials, TEM images showed that particles in the size ranges listed above for SEM did not appear to maintain their integrity in aqueous solution. Particles that adhered to the grid surface and subjected to negative staining were found to be much smaller, either in the range of 15 to 20 nm or larger (120 to 500 nm). Some of the larger sized particles had uneven borders and appeared to have been fractured. The most direct means of subjecting the same particles to analysis using TEM would necessitate suspending them in solution so they would adhere to carbon-stabilized Formvar-coated TEM grids. This solution
could either be aqueous or composed of a mild solvent such as ethanol that would not
dissolve the thin Formvar film that creates transparent windows over the grid
openings. Therefore, additional TEM imaging, using other solvents or methodologies,
may have generated more consistent results.

![TEM micrographs of 2-APri-ZnCl\textsubscript{2} complex encapsulate LC sample.](image)

3.4.1.2 Stability of the microencapsulated 2-APri-ZnCl\textsubscript{2} complex during storage

Due to the nature and limitations of the spray chilling encapsulation technique
there are three main structures that may be present in the resulting microencapsulates,
and hence affect the storage data. First, hydrophilic crystalline powder could be
exposed outside of the wall carrier, as shown in Fig 3.3, because spray chilling
encapsulation produces a matrix type encapsulate. Exposure of these exposed crystals
to high relative humidity (moisture) and other factors could be detrimental to the
stability of the “core material”. Therefore, it is desirable that the core material (flavor
complex) be completely contained within the carrier matrix. Secondly, polymorphic
changes in the lipid (Ubbink, 2002) can affect the physical characteristic of the wall;
hence its stability. Unstable alpha crystalline structure is a less structurally favorable
wall material and may facilitate premature release of the core ingredient and/or increase its vulnerability to the effects of environment exposure. Thirdly, hydrophilic compounds may form liquid flavor inclusions (Ubbink, 2002), thus allowing for loss of free ligand (2Apri) via diffusion and evaporation processes.

Fig. 3.3 SEM of microencapsulation containing 2-APri-ZnCl₂ outside lipid matrix

Different spray chilling trials were performed with hydrogenated vegetable oil, containing 5% (loading) of the complex (Table 3.1 and 3.2). The objective was to determine if encapsulates obtained from collection sites LC and SV exhibited differences with respect to morphological characteristics and storage stabilities.
**Table 3.1** Stability of encapsulated 2-acetylpyridine ZnCl$_2$ complex during 70 days storage 25°C *

<table>
<thead>
<tr>
<th>2APri-ZnCl$_2$ Complex/Hydro KoteC-Spray Chilling Encapsulant</th>
<th>Time (d)</th>
<th>Trial 1 (%)</th>
<th>Trial 2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC$^a$</td>
<td>0</td>
<td>1.675±0.033</td>
<td>1.305±0.015</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.707±0.028</td>
<td>0.827±0.038</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>1.547±0.046</td>
<td>0.819±0.014</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.492±0.073</td>
<td>0.827±0.006</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>1.347±0.008</td>
<td>0.776±0.016</td>
</tr>
</tbody>
</table>

* Values are expressed as the mean ± standard deviation of two trials and duplicates were analyzed in each trial.

$^a$ Lower Chamber (LC) and $^b$ Sample Vessel (SV) microencapsulated samples.

**Table 3.2** Stability of encapsulated 2-acetylpyridine ZnCl$_2$ complex during 70 days storage 25°C *

<table>
<thead>
<tr>
<th>2APri-ZnCl$_2$ Complex/Hydro KoteC-Spray Chilling Encapsulant</th>
<th>Time (d)</th>
<th>Trial 1 (%)</th>
<th>Trial 2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV$^b$</td>
<td>0</td>
<td>1.432±0.498</td>
<td>1.062±0.009</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.913±0.012</td>
<td>0.511±0.003</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>1.322±0.068</td>
<td>0.490±0.000</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.250±0.048</td>
<td>0.496±0.014</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>1.018±0.048</td>
<td>0.461±0.002</td>
</tr>
</tbody>
</table>

Storage analysis at ambient temperature showed loading (complex) retentions of approximately 80% and 59% in trials 1 and 2, respectively, in LC samples at day 70. Stability study results showed that the microencapsulated 2-APri-ZnCl$_2$ retentions were dramatically different between the two trials (Fig 3.4). This is thought to be due
to a variance in the operational parameters of the spray chiller. For example, 1) polymorphic changes may have been affected during one of the stages in cooling of the particles (Fig 3.5), and 2) expulsion of crystals (complex) from the lipid matrix could have occurred, making the complex more prone to environmental factors.

Fig 3.4. Retention of 2-Apri-ZnCl$_2$ encapsulate Storage experiment 0, 14,21,30,70 days at 25°C (mean±SD, n=2)
3.5 Conclusions

According to SEM imaging, spray-chilling encapsulation of 2-APri-ZnCl$_2$ complex in trials 1 and 2 formed microencapsulates of desirable morphological characteristics (particle size, smoothness, and spherical shape). In addition, SEM images showed that storage (14 vs. 70 days) affected the morphological characteristics of the microencapsulates, which were possibly caused by temperature fluctuation causing a type of “fat bloom” effect as indicated an alpha crystalline structure in the wall carrier. Storage analysis at ambient temperature showed loading (complex) retentions of approximately 80% and 59% in trials 1 and 2, respectively, in LC samples at day 70. The differences between the trials is thought to be due to the variability in the parameters of spray chilling process (differences in cooling of
microparticles could lead to variation in the alpha crystalline structure of the wall carrier structure).

Results of this study indicate that spray-chilling encapsulation may be a viable technique for the primary protection of moisture sensitive flavor compound-zinc ion complexes due to the hydrophobic nature of the shell material. Additional trials should be made in order to decrease variability in the initial loading (%) of the flavor compound-zinc ion complexes and to reduce differences in retentions affected by less-desired polymorphic crystalline structure. To reduce these effects a secondary coating to include all of crystalline complex in a reservoir type matrix might be used. Consequently, spray chilling encapsulation technique, with the appropriate adjustments, may be useful in stabilizing flavor compound-zinc ion complexes and enabling their use in industrial food applications (e.g., bakery, ice cream, and soups).
3.6 References


CHAPTER 4
SUMMARY AND CONCLUSIONS

In the present study we aimed to investigate the feasibility of utilizing a spray chilling encapsulation technique to protect a flavor compound-zinc ion complex, 2-acetylpyridine-ZnCl₂ (2-APri-ZnCl₂). 2-APri-ZnCl₂ was loaded at 5% (w/w) in Hydro-KoteC®, a commercial vegetable stearin. Samples of encapsulated 2-APri-ZnCl₂ complex from two different trials were collected from two different compartments (LC and SV) of the spray chiller apparatus and were analyzed for morphological characteristics and flavor stability. Stability data showed differences in flavor retention between trials 1 and 2 (80% and 59%, respectively) at day 70. Samples from LC and SV were subjected to SEM and TEM which showed the morphological characteristics of the encapsulates. Images from LC and SV were qualitatively different as shown by their SEM images. The samples from LC showed more homogeneity particle size and a smoother surface, which suggests good flowability characteristics at 14 days. Samples from the two trials were similar in that the spherical shape. Additional SEM images were taken at 70 days of storage, the spherical shape microencapsulates were still observed across all samples. However, it exposed a “fat bloom” characteristic which is associated to less robust crystalline lipid structure(α) in the wall material; hence, the likelihood of crystals complexes exposure to environment is expected. Despite the technical hurdles to reproducing the process conditions, specifically in the feeding process; reproducibility (SD±0.08, 0.016) of the loading was shown within each encapsulation trial (1 and 2)
respectively. Understanding of the operation parameters of spray chilling encapsulation as well as the morphological changes of lipid wall carriers are key to the encapsulation efficiency and extension of shelf-life of 2-APri-ZnCl₂ complex and could ultimately aid instable zinc ion aroma compound complexes protection by means of encapsulation technique.

Preliminary results from microencapsulates derived from this study suggest the method is feasible for the encapsulation of unstable molecules such as 2AP (ZnI₂ and ZnI₂). Their morphological characteristics (particle size, spherical shape) and hydrophobic nature would enable them to be applied in food industry (e.g. bakery, ice cream, soups, etc).

Additional studies should be done in order to address the stability issues; e.g. different lipid matrices (e.g. waxes, oils) should be studied. Furthermore, the influence of storage conditions such as temperature and relative humidity should be studied to determine the range of ideal temperature and predict its feasibility in food application. In addition to this study, a secondary protection may be used. Spray coating technique provides a reservoir matrix that could aid to entrap the complex crystal at lipid matrix surface that are prone to accelerate degradation of microparticles and/or enhance polymorphism. Needless to say cost constraints are to be considered before this could be considered an attractive flavoring material for food applications.
APPENDIX A RESPONSE FACTORS

Response Factor 2-Acetylpircidine (GC-FID)

(IS and APri)

<table>
<thead>
<tr>
<th></th>
<th>Target</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard:</td>
<td>2-acetypyridine</td>
<td>Collidine</td>
</tr>
<tr>
<td>Mfg/Reference:</td>
<td>Sigma-Aldrich</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Purity:</td>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cncn of Apri (ug/mL)</th>
<th>Cncn of I.S. (ug/mL)</th>
<th>Area Apri</th>
<th>Area I.S.</th>
<th>Area Ratio</th>
<th>Cncn Ratio</th>
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<td>0.198</td>
</tr>
</tbody>
</table>

Slope= 0.7405; R² = 1.3504

a. 2-Apri 403mg in 25mL Ethyl Ether
b. Internal Standard (I.S.)=Collidine=16150ug/mL(in ether)
c. 1µL solvent was injected to GC-FID.
Response Factor of 2-Acetylpyridine (GC-FID)
(IS/APri/Buffer)

<table>
<thead>
<tr>
<th>Target</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-acetylpyridine</td>
<td>Collidine</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cncn of Apri (ug/mL)</th>
<th>Cncn of I.S. (ug/mL)</th>
<th>Area Apri</th>
<th>Area I.S.</th>
<th>Area Ratio</th>
<th>Cncn Ratio</th>
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<td>797739</td>
<td>0.144</td>
<td>0.198</td>
</tr>
</tbody>
</table>

Slope = 0.7371; R² = 1.356

a. 2-Apri 403mg in 25mL Ethyl Ether
b. Internal Standard (I.S.)=Collidine=16150µg/mL (in ether)
c. 2-acetylpyridine+buffer(0.5mL)+IS(0.5mL), vortex 5min, then centrifuge(3000rpmx5min), 1μL solvent was injected to GC-FID
Response Factor of 2-Acetylpypyridine (GC-FID)
(IS/APri/Buffer/HKC)

<table>
<thead>
<tr>
<th>Standard:</th>
<th>Target</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mfg/Reference:</td>
<td>2-acetylpypyridine</td>
<td>Collidine</td>
</tr>
<tr>
<td>Purity:</td>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Cncn of Apri (ug/mL)</th>
<th>Cncn of I.S. (ug/mL)</th>
<th>Area Apri</th>
<th>Area I.S.</th>
<th>Area Ratio</th>
<th>Cncn Ratio</th>
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<td>123,406.00</td>
<td>878378</td>
<td>0.140</td>
<td>0.198</td>
</tr>
</tbody>
</table>

Slope = 0.7031; R² = 1.422

a. 2-Apri 403mg in 25mL Ethyl Ether
b. Internal Standard (I.S.)=Collidine=16150ug/mL(in ether)
c. 2-acetylpyridine+buffer(0.5mL)+HKC(15mg)+IS(0.5mL), vortex 5min, then centrifuge(3000rpmx5min), 1µL solvent was injected to GC-FID.
APPENDIX B SEM MICROGRAPHS

SEM Micrographs

Trial 1 Lower Chamber
SEM Micrographs

Trial 2 Lower Chamber
SEM Micrographs

Trial 1 Sample Vessel
SEM Micrographs

Trial 2 Sample Vessel
APPENDIX C TEM MICROGRAPHS

TEM Micrographs

Trial 1 Lower Chamber
TEM Micrographs

Trial 2 Lower Chamber
APPENDIX D TAGs CRYSTAL STRUCTURE

(Cross sectional diagram)

\( \alpha \) polymorph (unstable) \hspace{2cm} \( \beta' \) polymorph (metastable)

\( \beta \) polymorph (stable)