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Aroma components in Vangueria infausta L. : Characterization of components using GC_MS and aroma loss during drying

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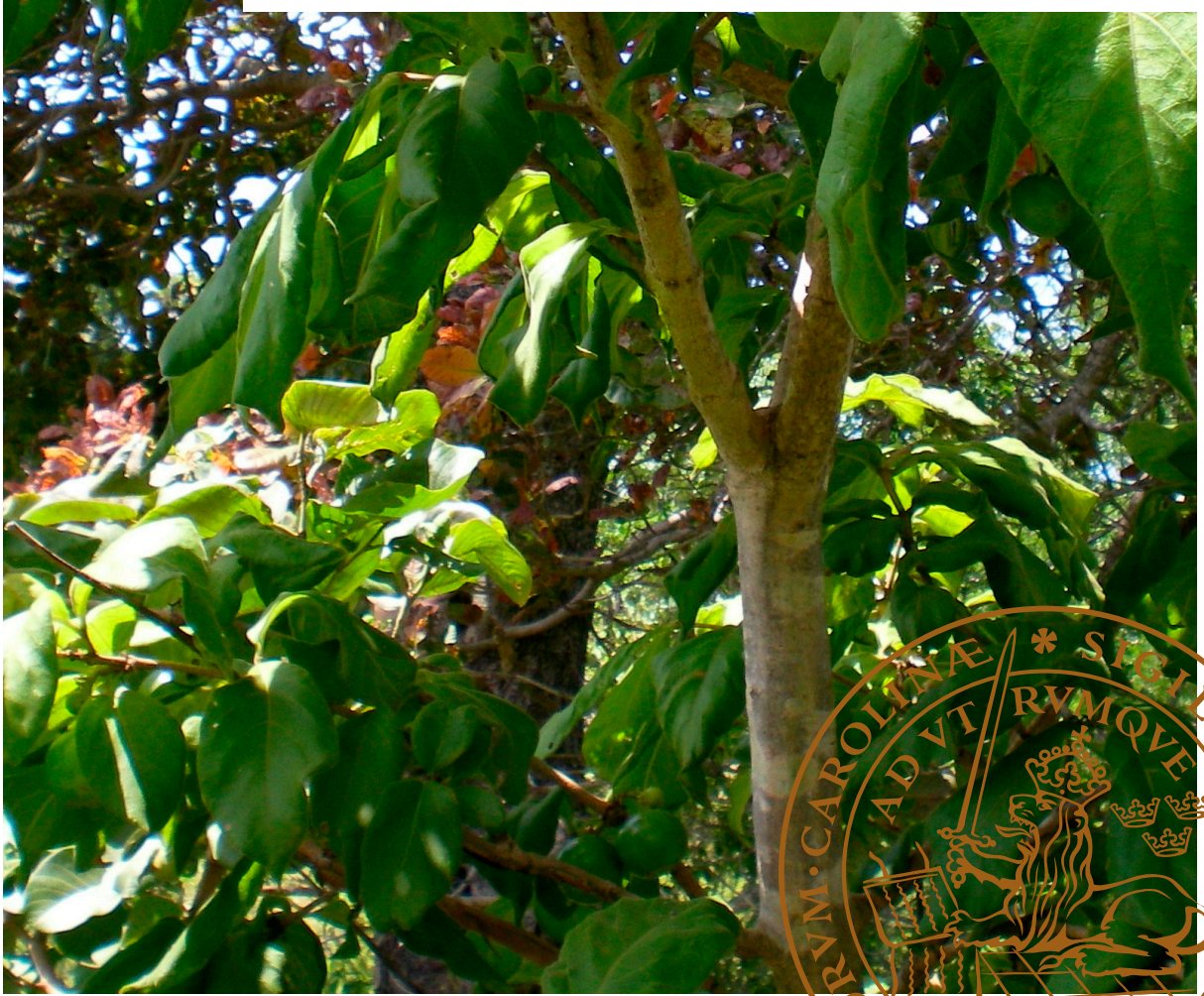

Universid E. Mondlane

Aroma components in *Vangueria infausta L.* :

Characterization of components using GC-MS

and

Aroma loss during drying.

Licentiate Thesis

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List of papers

Paper I. Raice, R. T., Sjoholm, I., Hong-lei, Wg, and Bergenstahl, B. (2013). ***Identification of volatile components isolated from Vangueria infausta (africana medlar) by using GC-MS*.** Submitted to the *Journal of Essential oil Research,* on 22/7/13; resubmitted on revised form on 17/12/2013.

Paper II. Raice, R. T., Chiau, E. F., Sjoholm, I. and Bergenstahl, B. (2014). ***Characterization of aroma loss after convective air-drying of Vangueria infausta L. (African medler) by using GC*.** Submitted to the *Journal of Food and Nutrition Science* on 6/6/2014; resubmitted on revised form on 10/6/2013.

The author's contribution to the papers

Paper I. The author performed the field work, designed the study together with co-authors, performed the lab work, evaluated the results together with co-authors and wrote the draft of the manuscript.

Paper II. The author performed the field work, designed the study together with co-authors, performed the lab work, evaluated the results together with co-authors and wrote the draft of the manuscript.

Related publications

Raice. R. T., Sjoholm, I., Da Cruz, J. F. and Bergenstahl, B. *(2011).* ***Identification of volatile components isolated from indigenous fruits of Mozambique: Maphilwa (Vangueria infausta)***; *Procedia food science, 1, 404-7*

Raice, R.T., Sjoholm, I., Francisco, J.C. and Bergenstahl, B. ***Identification of volatile components isolated from indigenous fruits of Mozambique: Maphilwa (Vangueria infausta)***; 11th International Congress of Engineering and Food; Presented at ICEF11 (May 22-26, 2011 – Athens, Greece) as paper FMS058.

Raice, R.T., Francisco, J.C., Sjoholm, I. and Bergenstahl, B. ***Characterization of indigenous fruits and study of aroma profile of indigenous fruits of Mozambique: Mapsinsha (Landolpia kraussi) and maphilwa (Vangueria infausta)***; Annual Meeting-Precess-Structure-Function relationships. Presented at EFFoST 2011 (November 9-11, 2011-Berlin, German) as paper P3.65

Abstract

This study focuses on the fruit of the African medlar (*Vangueria infausta* L., family *Rubiaceae).*

The investigation included the development of an extraction procedure of volatile components from the fruit matrix, a purification step, separation, identification and quantification.

Initial experiments showed that some components, especially sugars, degrads during the heating in the GC analysis, producing furfural, hydroxylmethylfurfural (HMF) and other volatiles. These compounds are obtained together with the native aroma components of the fruit making the analyses difficult.

We developed a procedure using a hydrophobic column with capability to retain the hydrophobic aroma components and eliminating sugars that may disturb the analyses.

The volatile components found in pulp is primarily: Hexanoic acid (30000-4000 µg/g, DM), octanoic acid (2000-200 µg/g, DM), ethyl hexanoate (400-40 µg/g, DM), ethyl octanoate (200-10 µg/g, DM), methyl hexanoate (60-8 µg/g, DM) and methyl octanoate (70-9 µg/g, DM). Based on the odor activity values (OAV) it can conclude that the odor of the fruit is mainly originated by ethyl hexanoate and ethyl octanoate. Fruity, sweet, floral and slight fatty are the most attributes that mark the aroma profile of studied fruit. The experiment was repeated using samples collected in different occasion (2010-2013).

During food processing and preservation of food especially fruits many attributes are affected including volatile components that are important attribute affecting palatability and consumer interest of food. The finding is that the principal aroma components of pulp are well preserved during the initial phase of drying (down to about a relative water activity 0.65). However, the aroma components are lost after a more extensive drying. A possible explanation of the volatilization is sugar crystallization that is to occur below a relative humidity of around 0.70 during the drying process.

The results highlighted, in somehow, the potential of *Vangueria infausta* as wild fruits to include in industrial food products. However, more studies are needed to improve the retention of volatile during drying. Encapsulation of these volatiles could help to sustainable utilization of diversity wild fruits growing in Mozambique.

Resumo popular

Frutos cultiváveis e domesticáveis constituem a dieta em termos de frutos da maior parte da região subsahariana de África onde também uma grande parte de frutos indígenas são consumidos.

Frutos indígenas como *Landolpia kirki*, *Ziziphus Mauritiana*, *Strychnos spinosa*, *Salacia kraussi*, *Anacardium accidentale*, *Vangueria infausta*, estão destribuidos um poco por toda a região subsahariana em especial em Moçambique, Botswana, Madagascar, South Africa, Zambia and Zimbabwe onde jogam um papel importante na dieta das comunidades rurais.

Este estudo inside se no fruto da *Vengueria infausta* vulgo maphilwa (na língua ronga em Maputo-Mocambique) também conhecido como African medlar nos falantes da língua inglesa. O fruto pertence botanicamente a família Rubiaceae. Quando maduro tem a côr castanha-alaranjada. O fruto tem em média 2-5 cm de diâmetro e contém cerca de 3-5 sementes. O fruto fresco tem sabor doce, ácido e nalgumas vezes azedo e o cheiro semelhante ao da medlar (*Mespilus sp.*) ou da maçã silvestre *(Malus sp.*). A polpa pode ser consumida fresca ou cozida. Pode ser usada para preparar sumo, jam, marmelada ou pudim por adição de água e açúcar ou ainda pode ser cozida em forma de massa. O fruto fresco pode também ser seco ao sol e guardado por mais tempo. Na região subsahariana o fruto amadurece durante abitualmente os meses the Fevereiro à Abril.

Antes de tudo é importante enfatizar que gosto e cheiro são provavelmente as chaves para garantir a qualidade e segurança do alimento. Por outro lado o agradável aroma dos frutos contribue para aferir maior valor na avaliação e segurança do fruto e estes jogam um papel importante na gastronomia.

Ainda assim alguns conceitos continuam mal entendidos quando estamos a tratar de campo de aromas. O assunto é de algum modo susceptível de ambiguidade na diferenciação entre cheiro e aroma. Cheiro é a combinação de sensações originadas através do gosto perceptível através de paladar na língua que detecta as sensaçôes básicas como o doce, acido, salgado e quente através da boca e o sentido the olfacto através do nariz. O termo aroma é mais utilizado para moléculas voláteis que são perceptíveis através dos receptores oflactoriais dentro do nariz.

Durante os anos passados, estudos relacionados com aspectos nutricionais de frutos indigenas de Moçambique foram feitos especialmente para maphilwa e reportam que estes frutos são ricos em fibras diéticas e açucares e têm alto teor de micronutrientes como minerais e vitaminas. Porém, poucos estudos foram feitos sobre a identificação de componentes da aroma da *Vangueria infausta*. Apenas foram encontados alguns estudos que versam a caracterização de componentes volatáis da *Vangueria madagascariensis* uma espécie similar a *Vangueria infausta*.

Ultimamente este fruto está atraindo muito interesse não só nas comunidades rurais como também na cidade onde o fruto é muitas vezes comercializado. Assim assumimos que o fruto pode ser útel tanto que o gosto e aroma são bem atraentes e apreciáveis. Daí o objectivo deste

trabalho é investigar o perfil de aromas dos frutos indigenas de Mocambique, particularmente de *Vangueria infausta* um dos frutos mais consumidos especialmente nas zonas rurais.

A investigação inclue um desenvolvimento de procedimento para extracção de componentes voláteis, purificação, separação identificação e quantificação.

Primeiras experiências mostraram que alguns componentes especialmente açúcares erão degradados durante o aquecimento dentro da coluna de GC e produziam furfural, hydroxylmethylfurfural e outros voláteis. Estes componentes eram obtidos juntamente com os aromas propriamente do fruto oque tornava a analise difícel.

Desenvolvemos um procedimento usando a coluna hidrofóbica que tem a capacidade de reter os componentes hidrofóbicos e libertar os hidrofílios através da lavagem com água.

Depois deste tratamento por coluna hidrofóbica os componentes eram dedectados no GC-FID.

Para identificação, o espectro de GC-FID foi comparado com o espectrómetro de mass providenciado pela Data base da biblioteca do Instituto Nacional the Referência e Tecnologia (NIST library). A identificação foi confirmada usindo materiais de referência.

O maior resultado que obtivemos deste procedimento foi que a extração tem de ser eficiente e isto constitue o ponto de partida desta investigação. Os componentes aromáticos podem ser extraidos com o etanol prior a mixtura de solventes como pentano e dietil eter. Os açúcares acompanham o extracto e tendem a degradar-se durante o aquecimento formando furfural, hidroximetilfurfural e dihidroximetilfurfural. Assim a inclusão de lavagem para remover todos os açúcares é crucial para a obtenção de material limpo adequado para ser analizado.

Os components aromáticos identificados na polpa da *Vangueria infausta* foram: Acido hexanoico (5600 µg/g, DM), acido octanoico (240 µg/g, DM), hexanoato de etil (44 µg/g, DM), octanoato de etil (13 µg/g, DM), hexanoato de metil (15 µg/g, DM) e octanoato de metil (12 µg/g, DM). Com base no valor de actividade do odor (OAV) é possivel concluir que o aroma do fruto é originado fundamentalmente pelo ethilhexanoate com pequena contribuição do etiloctanoate. Cheiro a fruta, flores, gosto adocicado e qualquer coisa como cheiro ransoso são as características mais salientes do aroma do fruto estudado. A experiência foi repetida usando frutos colhidos em diferente anos (2010-2013). Os resultados não mostraram grande variação.

Durante o processamento e conservação de alimentos especialmente frutos muitos atributos são afectados inclusive os componentes voláteis que são uma importante característica que afecta o paladar e o interesse do consumidor por determinado alimento. De modo a aprofundar este aspecto também foram feitas uma gama de experiências convista a avaliar o efeito da secagem na retenção de aromas da *Vanguera infausta.* usando um forno de secagem a ar e depois GC. A amostra foi seca a 80 °C, a velocidade de ar de 3 m/s durante diferentes tempos de secagem no intervalo de 60-420 minutos. O resultado obtido foi que todos os principais aroma previamente identificados eram retidos até quando a actividade da água na amostra fosse cerca de 0.65 oque corresponde até aproximadamente 240-300 minutos de secagem. Notou-se que os componentes aromáticos se perdem com a secagem prolongada até

mais ao menos quando a actividade da água cai abaixo de 0.65. A plausivel explicação desta volatilização de aromas pode ser o facto da cristalização de açúcares que assume se ocorrer quando a humidade relative durante o processo de secagem atinge cerca de 0.70.

Os resultdos evidenciam de algum modo a potencialidade da *Vangueria infausta* como fruto a ser incluso no processamento industrial de alimentos. Seja como for, mais estudos são necessários para melhorar a retenção de aromas durante a secagem. Acreditamos que a encapsulação desses voláteis pode ajudar para uma sustentável utilização da diversidade de frutos indígenas que se desenvolvem em Moçambique.

Contents

Abbreviations

IS----------internal standard
HMF----------Hydroximethylfurfural
OAV----------Odor activity value
GC-MS----------Gas chromatography mass spectrometry
StpTMC$_{18}$ ----------Hydrophobic column C$_{18}$
FID----------Fused Ionisation Detector
HRGC----------High Resolution Gas Chromatography
LRGC----------Low-resolution

1. Introduction

Food preservation with the aim of obtaining a desirable quality is a big challenge to confront. During food processing and the preservation of food, especially fruits, many attributes are affected including volatile components that are important attributes affecting palatability and consumer interest of food. Taste and smell are probably crucial for assessing the quality and safety of foods. Nevertheless, some concepts are still misunderstood when we are dealing with a volatile field. The issue is somehow susceptible to ambiguity on differentiation between flavor and aroma. Flavor is a combination of sensory inputs derived from taste or gustatory perception on the tongue that detects basic sensations such as sweet, bitter, salt, sour and umami together with sensations from the mouth (pungency) and olfactory perception from the nose. On the other hand, the term aroma is mostly attributed to the volatile molecules that are perceived by olfactory receptors in the nose.

The pleasant fragrance of fruit contributes to the added value for the evaluation and assessment of fruits and plays an important role on gastronomy (Kahkonen, et al., 1999; Kwang-Geun and Shibamoto, 2002). Furthermore, available studies show that the volatile chemicals present in leaves, flowers and fruits have also been widely used in aromatherapy since ancient times, suggesting that they impart some beneficial health effects (Kwang-Geun and Takayuki, 2002).

Rather than merely discussing the concepts, our interest has been to investigate the aroma components in selected indigenous fruits. Wild fruits like *Landolpia kirki*, *Ziziphus Mauritiana*, *Strychnos spinosa*, *Salacia kraussi*, *Anacardium accidentale*, and *Vangueria infausta*, are distributed over Southern African countries, for instance in Mozambique, Botswana, Madagascar, South Africa, Zambia and Zimbabwe, and play an important part, one way or another, in the diet in rural areas (Laverdière and Mateke, 2002; Amorteifio and Maosase, 2006; Styger et al., 1999).

A few investigations of the nutritional aspects were carried out which showed that these fruits are rich in dietary fiber and sugars and have a high content of micronutrients such as mineral and vitamins, (Magaia, 2013; Amorteifio and Maosase, 2006).

Vangueria infausta belongs to the family Rubiaceae. It is commonly called African medlar in English or maphilwa in Ronga (local language in southern Mozambique). The fruit is usually harvested in February to April. It is brownish orange when ripe and has a spherical shape. The size is about 2-5 cm in diameter and the fruit contains 3-5 seeds. The fresh fruit is sweet and tastes like green apple, somewhat like pineapple. Rural populations eat the pulp fresh, cooked or dried. They also prepare juice, jam and puddings by adding water and sugar or cooked to make marmalade. The pulp can also be fermented to produce alcoholic drinks. Often the fruit is dried in the sun and then stored for a long time. Lately the fruit has attracted interest not only in rural areas but also in the cities where it is very often brought and commercialized. Thus, our assumption is that the fruit may be useful and the taste and aroma profile attractive and appreciated. However, we have not found reports about the identification and quantification of aroma components in *Vangueria infausta*. Only one study of the characterization of volatile components of the closely related *Vangueria madagascariensis*

has been found (Pino et al., 2004). Therefore, the aim of this work is to investigate the aroma profile of wild fruits of Mozambique particularly the *Vangueira infausta*, one of the most consumed especially in rural areas.

The investigation included the development of an extraction procedure of volatile components from the fruit matrix, a purification step, separation, identification and quantification. The extraction is carried out using ethanol. The role of ethanol is to help us to disintegrate the pulp material, to penetrate into the tissue structure and to release in water poorly soluble aroma substances. Initial experiments showed that some components, especially sugars, are degraded during the heating in the Gas Chromatography (GC) analysis producing furfural, hydroxyl methyl furfural (HMF) and other volatiles. These compounds are obtained together with the native aroma components of the fruit making the analysis difficult. Thus, prior to separation by GC it was necessary to purify the primary extract from sugars. We developed a procedure using a hydrophobic column with a capability to retain the hydrophobic aroma components and wash the hydrophilic components using water. The collected aromas were released from the column using a mixture of pentane and diethyl ether. After the refining step the extract was injected into the GC. In the GC the aroma components were separated based on their retention times. After the column the signal was detected using a flame ionization detector with a very high sensitivity. For the identification a mass spectrometer mounted as an online detector at the GC provided spectra that were compared with data from the National Institute of Standards and Technology (NIST) library. The identification was verified using standards. The final step was to calculate the quantitative amount related to each peak, taking into account the peak areas of components related to the internal standard.

Furthermore, a set of experiments was performed to evaluate the effect of drying on the retention of aroma. The finding is that the volatiles of *Vangueria infausta* are lost during the drying process although a plausible explanation is still to be clarified through further studies involving suitable models such as pectin and microcrystalline cellulose.

2. Objectives

2.1 General objective:

The main objective of this study was to understand the aroma composition in the fruit of *Vangueria infausta* (African medlar) and retention during the processing of wild fruits of Mozambique.

2.2. Specific objectives:

To develop a suitable method for aroma extraction from *Vangueria infausta*

To identify and characterize the aroma components of *Vangueria infausta*

To understand the aroma retention phenomena during the processing of *Vangueira infausta*

3. Background

3.1 Aroma compounds found in common fruits

The aroma compounds present in the fruit mostly originate from a mother plant and are a consequence of numerous agricultural factors such as soil types, cultivation conditions, growth and climate as well as biochemical factors like the development of enzymes during the maturation stage, metabolic pathways during harvest and methods of harvesting (Cheetham, 2010; Wright, 2010 and Kaewtathip, et al., 2012). In Table 1 common aromas from common fruits are listed as typical examples of common structures.

Table 1. Flavor compounds found in most common fruits.

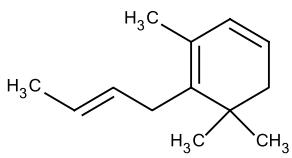
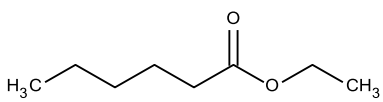
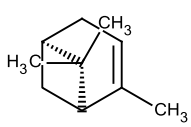
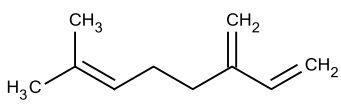
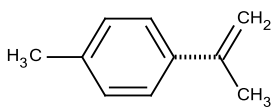
Common Fruit (Scientific name)	**Main aroma compound**	**Chemical structure**	**bp (°C)**	**log P**	**Literat Source**
Apples *(Malus domestica Borkh)*	β-damascenone		167	N/f	Qian, Xu and (2007).
Pears *(Pyrus serotina L.)*	Ethylhexanoate		166	2.3	Takeoka at al., (1992).
Orange *(Citrus sinensis* Osbeck*)*	α-pinene		155	4.37	Shaw, and Moshonas, (1994).
	Myrcene		167	4.58	
Lemon *(Citrus Limon L.)*	Limonene		177	4.45	Moufida and Marzouk, (2003)

Table 1 (Continuation)

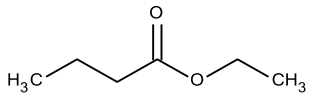
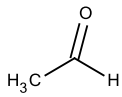
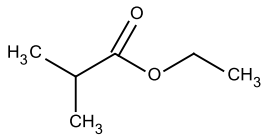
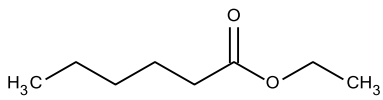
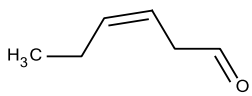
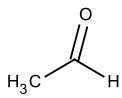
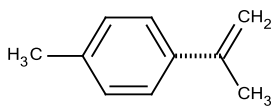
Mango *(Mangifera indica L.)*	Ethyl butyrate		120	1.85	Pino and Mesa, (2006). Adedeji, et al., (1992).
	Acetaldehyde		20	0.6	
	Ethyl 2-methyl propanoate		110	1.77	
Banana *(Musa sapientum L.)*	Ethyl hexanoate		166	2.3	Jordan, et al (2001).
	Cis-3-hexenal		126	N/f	
Grapefruit *(Citrus paradisi Macfayden*)	Acetaldehyde		20	0.6	Schieberle and Buettner, (2001).
	Limonene		177	4.45	

Table 1. (Continuation)

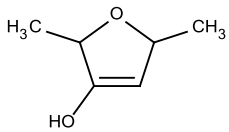
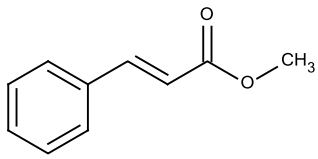
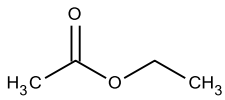
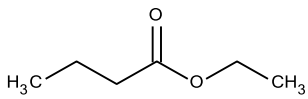
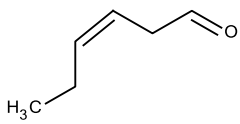
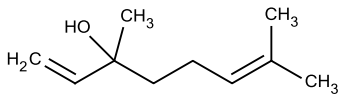
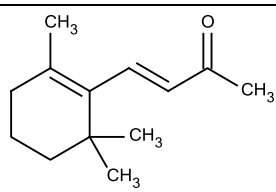
Strawberry *(Fragaria ananassa)*	Cis-3-hexenol		-61	1.61	Vaughn, et al., (1993)
	4-hydroxy-2.5-dimethylfurane -3-one		N/f	N/f	
	Methyl Cinnamate		261	N/f	
Plums *(Spondias mombins L.)*	Ethyl acetate		77	0.86	Sagrero-Nieves, And De Pooter, (1992).
	Ethyl butyrate		120	1.85	
Guava *(Psidium guajava L.)*	Cis-3-hexenal		126	N/f	Steinhaus, et al., (2009).
Apricot *(Prunus armeniaca L.)*	Linalool		198	3.28	Takeoka, et al., (1990).
Peach *(Prunus persica L.)*	β-ionone		126	N/f	Takeoka, et al., (1990).

Table 1. (Contination)

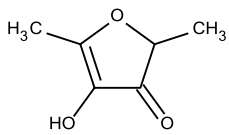
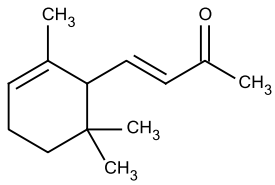
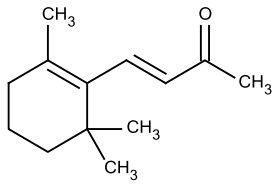
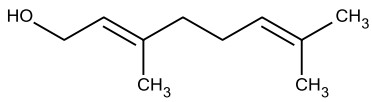
Pineapples *(Ananas comosus L.)*	4-hydroxy-2.5-dimethyl-3-(2H)-furanone		N/f	N/f	Wu et al., (1991).
Raspberry *(Rubus idaeus L.)*	α-ionone		N/f	N/f	Larsen and Lewis (1991).
	β-ionone		126	2.10	
	Geraniol (IS)		229	3.28	

Table 1 shows that a majority of these aromas have boiling points in a range of 100 up to 200 C and they also show a hydrophobic character which is signalized as high values of the log(P), the distribution coefficient between octanol and water. We assume that by taking into account these two parameters and also the use of non-polar extraction solvents it can be possible to set a GC procedure within a certain temperature range which may provide a reliable aroma profile.

4. Methods of identification of aroma

Today numerous techniques are available for analytical purposes. Unfortunately, only very few of them are really suitable for flavor research. The extremely low amounts needed for the analysis of aroma (10^{-9} g or less), mostly obtained after tremendous purification and concentration steps, makes the number of choices very limited when comparable to the amount (about 10^{-4} g) needed to perform analysis by any classical identification techniques. GC-MS are the most useful for aroma component identification although other methods such as Infrared (IR), Nuclear Magnetic Resonance (NMR) and Electronic Nose (EN) may also be used (Reineccius, 2010).

4.1 Gas chromatography

Gas chromatography (GC) is a basic tool for the analysis of aroma. In this study the GC 6890 (from Agilent Technology) was used, more details of which will be considered in the course of this thesis (papers I and II). However, GC is used for separation in an analysis of substances susceptible to vaporization without decomposition. The main characteristic of GC is to have a mobile phase, usually an inert gas (helium or nitrogen), and a stationary phase, often a microscopic layer comprised of polymer on an inert solid inside a column. The main stationary phases are cyanopropylsiloxane, octadecyl groups, and sulphoxyl groups. The boiling point of the substance and the interaction between the substances being analyzed with the stationary phase is a key factor for separate elution of the component at specific times known as retention times which, is later compared with similar reference materials. The analysis starts by injecting (using a microsyringe) the sample into the GC column where the mobile phase is continuously moving at a certain velocity. The substances are retained separately by adsorption that depends on the size and type of molecule, and released from the column at different times (retention time). This process is monitored by a detector which allows for the determining of not only the time that the substance leaves the column but also the amount of the substance through the peak area or intensity on the correspondent chromatogram. Commonly, the substances are qualitatively determined by the order in which they are released from the column.

4.2 Mass spectrometry

Mass spectrometry (MS) is a second technique following the GC. While the GC is performed to separate the components the MS is the mass-selective GC detector responsible for the identification of these components. Its possibility to connect to the GC, its tremendous sensitivity measured in the hundreds of pictograms and also its highest capability to give more structural information make the MS the first choice when the issue is the identification of volatility.

The MS is operated in selective ion-detection mode (quadrupole) to select ions at very short mass intervals. This capability allows for separately quantifying two components simultaneously eluted from GC, for example isotope counterparts (Reineccius, 2010).

It should be emphasized that an MS detector is generally more sensitive than Flame Ionization Detection (FID) and this high ion selectivity is the key factor for achieving excellent results in the analysis of aroma extracts. Obviously, poor GC resolution or a limited sample may make the interpretation of MS chromatogram difficult. However, the good flavor extraction still determinant to also good MS results.

Mass spectrometer apparatuses may be divided into two main groups: low-resolution (LR) and high-resolution (HR) instruments. The LR instruments provide sufficiently accurate mass measurements to the nearly whole mass unit and so can allow determination of elemental composition while, since many elements or combinations of elements may give the same unit mass (isomers), LR may give only molecular mass. LRs were the most commonly used

instruments a few years ago due to their easy operation and cheap acquisition as compared to HR equipment.

The use of GC-MS is today spread world-wide and allows for the designing of more understandable spectral libraries and matching systems to allow for the quick and secure identification of flavor compounds.

5. Retention time

Retention time is the time that the compound spends from the injection until its detection after passing the heated gas chromatography column. The boiling point of the substances to be separated and the affinity to the stationary phase within the column plays an important role in the retention of the substances. In this study the DB-225 column was used, which is a polycyanopropylphenyldimetylsiloxane composed by 50% of cyanopropylphenylsiloxane groups. This stationary phase is useful in GC due its high temperature stability that makes it suitable for wide temperature ranges, chemical inertness and good film forming properties (Wiridena et al., 2001). The high thermal stability is beneficial for the experiment since we assume that most of the volatiles are high boiling substances. On the other hand, the large quantity of the cyanopropylphenylsiloxane groups makes possible more cohesion with volatile compounds which is crucial for selective separation as the temperature increases inside the column. However, the specific conditions between experiments may vary and factors such as flow rate of the carrier gas, length of the column, and gas pressures bring uncertainty to retention time values and make the absolute retention value an uninteresting measure. A way to overcome this limitation is the use of relative indices (RI) or Kovat's indices (Takács, et al., 1971; Acree, 2001; Majlát, et al., 1974; Kondjoyan and Berdague, 1996). Relative retention indices represent the retention time of an unknown compound relative to an n-paraffin or ethyl esters series. Usually RI and its thermal sensitivity are directly dependent on the column polarity. It is assumed that an appropriate stationary phase column and a good range of temperature programs are the key factors for accomplishing a confident separation and identification of the compounds by comparison of the observed RI with a similar compound from a spectral library (Wei-chuan et al., 1995). However the use of only RI is, in many cases, not sufficient for compound identification. Thus, additional data obtained from the peaks, such as mass spectrum is needed (Wiridena, 2001, Reineccius, 2010).

6. Threshold and odor activity value

An important aspect of aroma analysis is the intensity of the perception. However, it is difficult to evaluate the intensity of an odor sensation since the perceived intensity depends on time, and the character may depend on the intensity of the exposure. The most common

approach is to measure the critical concentration when the perception can be recognized. This limit is known by a threshold that is defined as a minimum concentration value at which an aroma can be active and sensorially perceived (Plotto, et al., 2008; Grosh, 1994; Rothe and Thomas, 1963). The structure also plays a prominent role with regards to flavor threshold. To give an idea: the 2-ethylhexyl 2-methyl-2-propenoate has a threshold of 0.02 µg/L in aqueous solution while the 2-ethylhexyl 2-propenoate only has 0.005 µg/L. This means, 4-fold difference is due to slight complexity of the first compound (Gemert, 2011, Cheetham, 2010).

The ratio of concentration by threshold is known by Odor activity value (OAV). Theoretically, any individual flavor component needs to be present at about ten times its threshold in order to have a significant flavoring effect (Preininger and Grosh, 2001). However, it is necessary to take into account that the perception of the flavor attributes (quality, character and intensity) are dependent on prior evaluator experience (regional, cultural, age) even for a majority of basic aroma sensations.

In practice, compounds with OAV>1 are considered as individual contributors to the smell although aromas perceived when sniffing the fruit are a combination of various compounds and could not be attributed to a unique component (Munoz et al., 2007). Even at concentrations below the odor threshold, the aroma compounds may produce an aroma that is perceived as a result of perceptual synergy that gives a specific impact of the fruit (Grosh, 2001, Gomez Garcia-Carpentero et al., 2011; Kaewtathip and charoenrein, 2012; Lopez et al., 2003; Ferreira et al., 2001). Numerous studies have been conducted to determine the minimum concentration of flavor that could be perceived by human olfactory sense. Large data collections are available. The table 2 shows the threshold values of some fruits measured in aqueous solution.

Table 2. Threshold values of some aroma compounds found in fruits.

Aroma Compound	Limonene	Myrcene	Hexanoic acid	Ethyl hexanoate	Methyl hexanoate	Methyl octanoate	Octanoic acid	Ethyl octanoate	α-Terpineol
Threshold (µg/g)	1000[b]	15[b]	3000[b]	1,0[a, b, c]	70[b]	200[a, b]	3000[b]	5[d]	330[b]

The superscripted lower character indicates the source from which the data was collected as follow: [a] - Odor threshold concentration in water (µg/g). From Monteiro-Calderón et al. (2010). [b] - Odor threshold concentration in water (µg/g). From Pino and Mesa (2006). [c] - Odor threshold concentration in water (µg/g). From Takeoka, et al. (1995). [d] -Odor threshold concentration in wine model (µg/g). From Ferreira et al. (2000), Gómez et al. (2012), Wu et al. (23), Qian and Wang (2005).

7. Aroma isolation from plant material

The aroma components may be distributed within all major parts of plants including fruits, roots and leaves. There are different methods which can be selected to isolate the aroma compounds from the tissue that we intend to analyze. The main principles of extraction are based on volatility or solubility of aroma compounds (Sides, et al., 2000, Wright, 2010). The close constraint is that also water is volatile. All suggested procedures represent compromises between different types of errors such as aroma losses, oxidation, thermal degradation, and contamination by artefacts and they may be time-consuming (Sides, et al., 2000).

7.1 Contamination by artefacts

During the handling of samples special attention has to be paid to ensure an extraction with the minimum amount of contamination possible from all sources. The use of lab gloves, for example, can prevent skin lipids from contaminating the sample. It is always recommended to utilize GC grade solvents and distilled water and to avoid the utilization of greases and rubber-based materials. It should also be highlighted that the heating should be moderate and, preferably, not more than 60°C because already at this temperature volatile material can be expected to evaporate from the sample. Another source of contamination due to heating is the risk for degradation of sugars to produce reaction products such as hydroxyl methyl furfural (HMF) and hydroxyl dimethyl furfural (HdMF) (Reineccius, 2010). However, heating may be necessary in order to inhibit enzymatic degradation, and to obtain an enzymatic release from various complex carbohydrate structures, as well as to disrupt the botanical microstructure.

7.2 Isolation of aroma based on volatility

The aroma components are characterized by exhibiting sufficient vapor pressure to be detectable by the olfactory system. One possibility to isolate aroma substances is to isolate them based on volatility. The most common techniques are Distillation, and Static and Dynamic headspace. The distillation methods are the oldest and most common techniques of aroma isolation from tissues.

7.2.1 Simultaneous distillation-solvent extraction is one of the most useful techniques for the isolation of aromas. The substance being analyzed is distilled, which is immediately followed by extraction with solvents using a coolant substance, often methanol, at 2°C or a water bath at 10°C, to prevent the rising of the temperature. Afterwards, the solvent is removed by using a Vigreux column (Núnez, 1984. The major advantage of the method is the low possibility of contamination by artefacts.

7.2.2. Static headspace is a method that consists of heating the food at certain temperatures sufficient to release vapor from a food into a closed compartment from which a sample is immediately taken by a syringe and directly injected into a GC (Attaie, 2009). This technique is used, for example, to obtain a profile of the most volatile compounds and the most abundant volatile during quality control trials where only major components need to be measured. The advantage of the methodology is the simple sample preparation and also that no concentration step is required. The signal intensity is a direct function of the thermodynamic activity of the aroma substance in the food matrix. A noted disadvantage is

the use of a very small amount of samples (not more than 1 ng/L) which may be difficult to handle until the next analyses step if we consider that usually the GC-FID and the MS do not detect less than 10^{-7} g/L and 10^{-5} g/L, respectively (Reineccius, 2010).

7.2.3 Dynamic headspace is also known as the headspace trapping, purge-and-trap or headspace concentration method consisting of purging the sample with gases, usually nitrogen and hydrogen followed by trapping with appropriate trapping agents such as activated charcoal and coconut charcoal. The role of these agents is to collect the aroma from the stream, which is later released into the heated GC column by adsorption. The activated charcoal is preferable due to its high affinity with most aroma compounds. However, further purification may be needed as also non-polar impurities may be trapped during the process.

7.3 Isolation of aroma based on solubility

7.3.1 Solvent extraction is another classical procedure which is applied for the isolation of aroma components through its solubility in secondary media. A majority of the aroma components (Table 1) are hydrophobic. The extraction includes a sequence of steps: placing the sample in contact with the solvent during a certain time, shaking the mixture moderately, repose, phase separation by using appropriate funnel, eventual drying by using anhydrous salts (Na_2SO_4), eventual aroma concentration by using a stream of nitrogen or a vapor rotator device or other cleaning efforts with the objective of eliminating other components that may disturb the analyses. Finally, the concentrated extract is analyzed by being injected into the GC apparatus (Reineccius, 2010). The main disadvantage of the method is, apart from the presence of lipids, the GC grade solvent needed and high precaution needed in the case of using supercritical fluid.

7.3.2 Supercritical CO2 technique is an extraction using a certain type of solvent system-a supercritical fluid. The most common supercritical fluid is supercritical CO_2 (at a pressure and temperature above critical conditions of the gas it changes into a supercritical condition) (Francisco da Cruz, 2002). The sample is placed in a chamber together with the supercritical fluid (CO_2) and is extracted. The supercritical fluids are described as "super solvents" (Hawthorne, 1990) although the extraction capacity is limited due to comparable low solubility (caused by the low density of the solvent). The main advantage is the good penetration capacity into the sample matrix, the possibility to change the pressure and temperature of the inner chamber and thereby the easy separation of the target substances from the extraction fluid. However, the sample size is very small and the cost of equipment is quite high due to the pressure requirement.

7.4 Solid-phase micro-extraction (SPME) is a technique for qualitative analyses. An inert fiber, coated with an adsorbent material, usually activated carbon of coconut charcoal, is immersed in a sample or put into contact with the headspace of the sample. The aroma components are adsorbed at the absorbent material and then later released and injected into the GC (Reineccius, 2010). The disadvantages are the low quantity of the adsorbent available for volatile adsorption, low phase ratio.

8. Experimental work for aroma analysis

8.1 General overview of analytical procedure

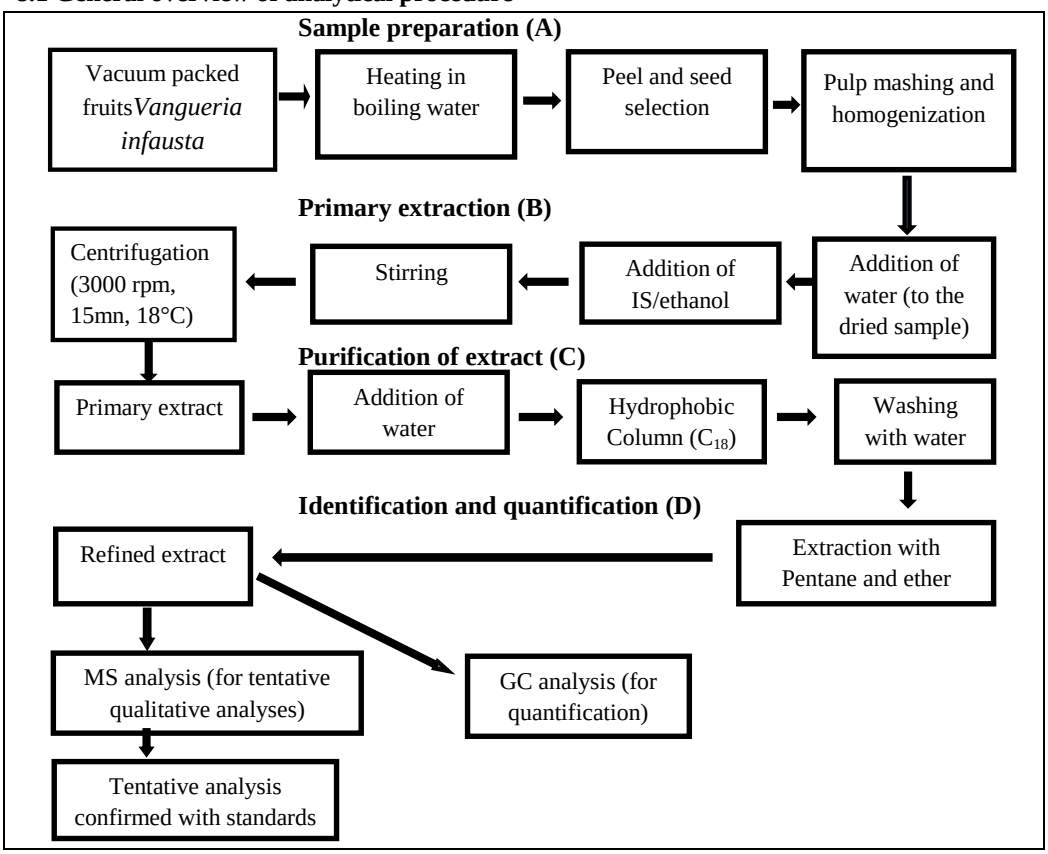


Figure 1. Purification of extract of *Vangueria infausta*

8.1.1 Sample, collection and preparation (A). Mature *Vangueria infausta* fruits have been collected from Bobole and Marracuene (Maputo, Mozambique) during maturation stage usually in March, during 2010-2012 seasons. Defect free fruits were selected, washed, vacuum-packed, sealed in plastic polyethylene bags and frozen at - 20 °C.

Before the experiment, the vacuum-packed samples were thawed in boiling water for 10 minutes in order to inactivate enzymes like lipoxygenase (LOX), hydroperoxidelyase (HPL) and alcohol dehydrogenase (ADH). The aim was to prevent degradation during the extraction that could bring uncertainty in the extraction process (Bai et al., 2011 and Baldwin et al., 2004). The fruits were allowed to cool. Peels and seeds were manually separated from the pulp. The pulp samples were disintegrated and homogenised by mushing with a clean spoon.

8.1.2 Primary extraction (B). The sample was macerated in a glass Erlenmeyer flask with 100 ml of ethanol containing internal standards of geraniol. A small amount of distilled water was added to the dried sample to allow for the disintegration of the matrix (for better extractability). The extraction was performed under continuous stirring using a magnetic stirrer, during 72 hours, in order to provide time for the extraction. The suspension was centrifuged at 3000 rpm for 15 min. Then the primary extract, the supernatant, was divided into three replicates with 10 ml in each.

Ethanol was the preferred solvent, due to its hydrophilic character, which is assumed to allow for better penetration into fruit tissue and allow for effective extraction of aromas enclosed in the tissue structure. Ethanol also denatures enzymes such as β-glucodidase which may prevent degradation during extraction time that could bring negative influences to the results (Bai, et al., 2011). An internal standard (geraniol) was added to the sample together with the ethanol in the initial mixing, as the liquid phase could not be quantitatively separated from the suspension.

Direct analyses of primary extracts have been evaluated. The GC chromatogram showed many peaks presenting products originating from sugar degradation reactions, mostly HMF and hydrocarbons (Figure 2). Similar substances have been reported as aroma components of *Vanguria madagascariensis* in a previous study by Pino and coworkers (Pino, 2004). A possible explanation of these results is that they originate from sugars that are decomposed in the GC column or injector system. In the literature it has been observed that sugars (glucose, fructose and sucrose) when extracted with a hydrocarbon phase (tetradecane and dodecane) and injected into the GC result in in the formation of HMF due to the degradation of sugars in the column (Cheetman, 2010). This inconsistency enforced us to include a purification step prior to the GC analysis with the aim of eliminating the sugars.

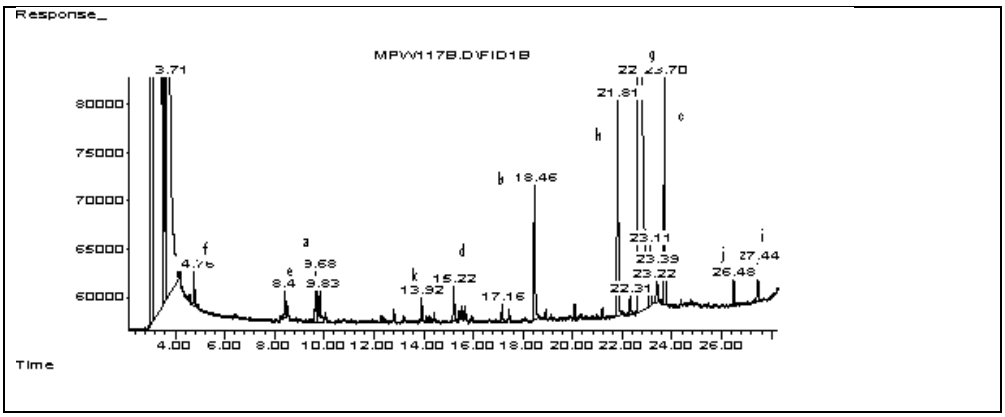


Figure 2. A chromatogram showing aroma compounds found in the primary extract of *Vangueria infausta:* The chromatogram shows peaks presenting products of sugar degradation like: a- furfural and c- 5-hydroximethylfurfural which were later confirmed by MS analysis

8.1.3 Purification of the extract (C). The purification in the extraction phase is a crucial step in aroma analysis (Hattab, et al., 2007). The purification is intended to eliminate or minimize the influence of the artefacts to the analyses. Previous works reported that material such silica, activated charcoal, coconut charcoal, siloxane, cyanopropylphenyl, dietylpolysiloxane are used for purification of aroma extracts (Lopez et al., 2003, Reineccius, 2010, Lopez et al., 1999, Gomez et al., 2011). In this work the objective of the purification step is mainly to remove saccharides from the extract to eliminate the formation of degradation products during the analyses. In this work the purification was carried out using hydrophobic C_{18} column. The hydrophobic volatile compounds are assumed to absorb during this step. The solvent phase is made somewhat more polar by adding water prior to the purification (30 ml of pure water was added to 10 ml of primary extract). To ensure the effective cleaning, the column was rinsed with 5 ml of water in order to eliminate remaining sugars and other low molecular-weight polar compounds. Then the aroma components were extracted with 10 ml of mixture of pentane and diethyl ether (1:1) through desorption from the hydrophobic column C_{18}. The column contains octadecyl unendcapped bonded silica (from Capitol Scientific Inc., Austin, Texas, USA). The elution is performed with a flow rate of 1 ml/min. The refined extract was collected, introduced on 2.0 ml vial to GC analysis.

8.1.4 Separation of the aroma components (D). GC analysis was carried using an Agilent GC 6890 (G1530), Network GC system, serial US10322054, purchased from J&W Scientific, USA. It is a bench-top instrument equipped with a fused capillary column (J&W DB225, 30m x 0.25mm, coating thickness 0.25 µm) containing hydrophobic stationary phase of cyanopropylphenyl-dietylpolysiloxane (50%). The inlet volume was set on split mode at split ratio 20:1 and split flow 12:1. The chromatogram conditions were set as follows: Injector and interface temperatures were 250 °C; the column temperature was held at 50 °C for 5 min, programmed at 3 °C/min to 200 °C and then held there for 3 min. Helium was used as carrier

gas at flow rate of 0.6 ml/min, 55.2 kPa. The hydrogen flows at 30 ml/min. The injector had a syringe size of 10µl, volume sample of 2.0 µl was injected three times, and n-hexane was used to wash five times. The FID chromatogram (Figure 3) reveals that there are about 10 components can be efficiently separated within 34 minutes. The number as well as the intensity of the peaks is much reduced and none of them represents the typical saccharides decomposition products such as furfurals were observed.

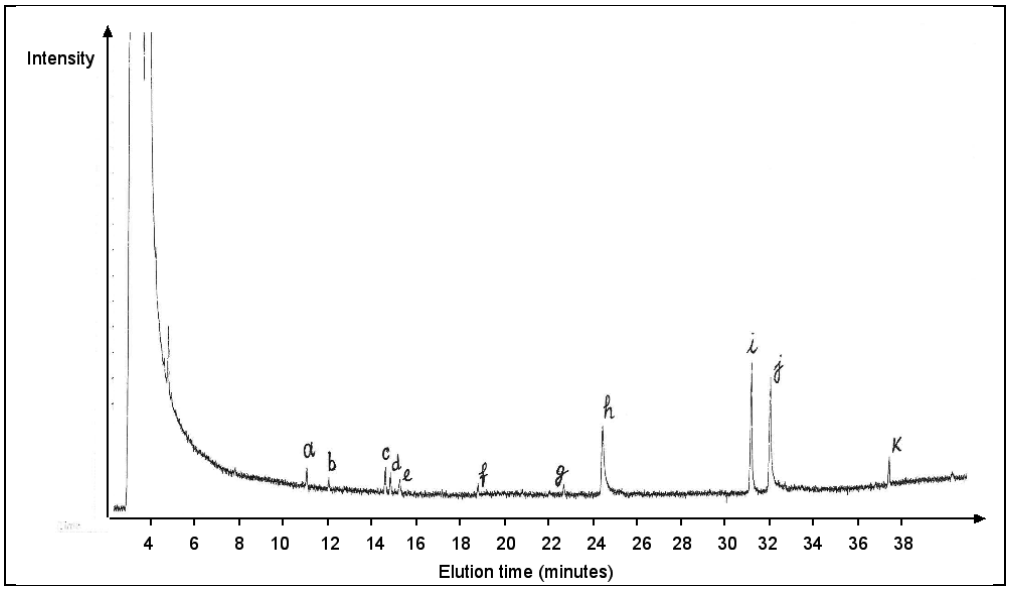


Figure 3. A chromatogram showing aroma compounds found in the extract of *Vangueria infausta* after cleaning through C_{18} column: a -methyl hexanoate, b -octane, c -hexanoic acid, d -ethyl hexanoate, e -disulphide, f -undecane, g -ethyl octanoate, h -octanoic acid, I -geraniol (IS), j -tridecane, k -16:0 methyl esters. The chromatogram was obtained using DB-225 column

The FID GC parameters were slight modified as follow: The inlet volume was set on split mode at split ratio (20:1), split flow (12:1) and injector and interface temperatures were 250 °C. All other parameters maintained unmodified. As outcome, this change reduced the noise and improves resolution to the chromatogram (Figure 4). The retention times of the target components (in each replicate) were comparable with that on for the reference material.

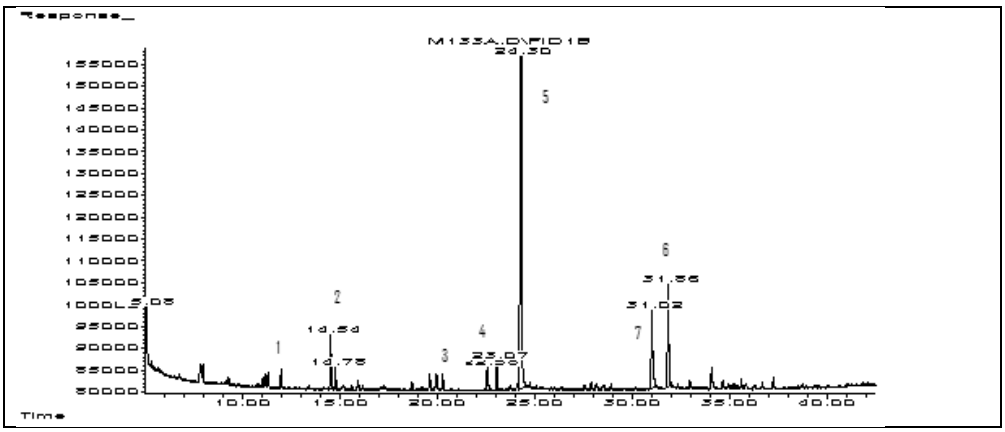


Figure 4. FID chromatogram of *Vangueria infausta* as obtained after cleaning with C_{18}, using a DB-225 column (30m x 0.25 mm x 0,25µm film thicken, split ratio 20:1 and flow ratio 12:1): 1-methylhexanoate (Rt=12.03 min), 2-ethylhexanoate (Rt=14.54 min), 3-methyloctanoate (Rt=20.28 min), 4- ethyloctanoate (22.58 min), 5-hexanoic acid (Rt=24.30 min), 6-octanoic acid (Rt=31.85 min), 7-geraniol (IS) (Rt=31.02 min).

8.1.5 Identification of the aroma components (D).

MS analysis was performed on an Agilent (Palo Alto CA, USA) 5972 mass selective detector together with an Agilent 6890 GC equipped with a HP-5MS capillary column (30 m x 0.25 mm i.d., SGE, Austin, TX, USA). The column was coupled in the electron ionization (EI) mode at 70 eV. The components were tentatively identified by matching obtained spectra against Wiley Spectral Library using the PBM logarithm. The identification was confirmed using pure standards. The retention times as well as the MS spectra were considered similar. The target components identified were: methyl hexanoate, ethyl hexanoate, methyl octanoate, ethyl octanoate, hexanoic acid and octanoic acid. To confirm this finding, GC-MS analyses using two different columns were carried out; one with high polarity Innowax, and one non-polar column HP-5. The GC-MS results from these two columns were in a different order and showed that co-elution was not a major source of misinterpretation and all peaks were in agreement with reference compounds. One particularity is that the use of highly polar columns results in poor resolution of fatty acids. Therefore, in order to avoid possible misinterpretations, further experiments with methanolysis conversion were performed on a DB-225 column from which chromatogram presented the fruit's potential aroma component along with other secondary substances such as hydrocarbons (undecane, tridecane), peroxides such as di-tert-butyl peroxide (DTBP) and 2,6-di-tert-butyl-4-methyl-phenol (BHT), and dissulfite. We believed that these products are probably impurities like finger prints, rubber tubes polymerization agents and stabilizers of the diethylether. For example, Figure 5 shows the fragment patterns for hexanoic acid and Table 3, the concentration of the components.

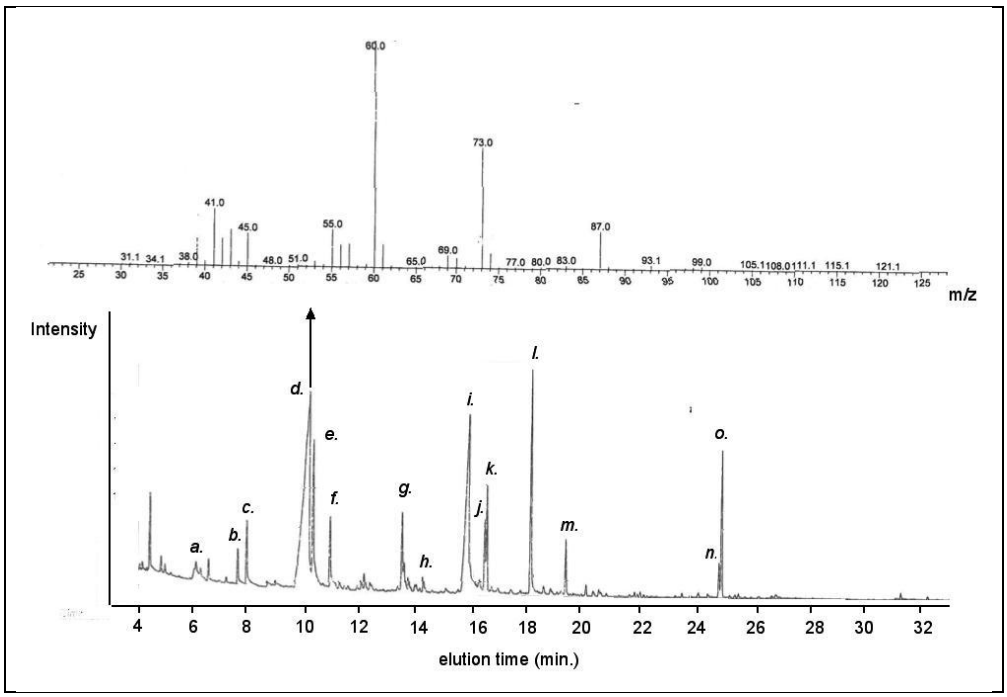


Figure 5. GC-MS analysis of the extract from *Vangueria infausta* using HP-5 column. The peak at the retention time of 10 minutes has been selected for displaying the MS pattern. The fragmentation pattern of the peak suggest that it is hexanoic acid. *a*-octane, *b*-butanol, *c*-methylhexanoate, *d*-hexanoic acid, *e*-ethylhexanoate, *f*-dissulfide, *g*-undecane, *h*-methyloctanoate, *i*-octanoic acid, *j*-ethyloctanoate, *k*-dodecane, *l*-geraniol (IS), *m*-tridecane, *n*-DTBP, *o*-BHT.

8.1.6 Quantification of the aroma components (E). The amount of each aroma compound was calculated relative to the dry matter (DM) of the samples considering the signal intensities of the reference compounds. We assume that through this procedure we eliminate dilution errors and variations in the extraction yield caused by solvent entrapment. However, errors caused by unfavourable distribution equilibriums in the first, and the second extraction respectively, cannot be compensated. The equations below were used for the aroma concentration calculation:

$$C_{aroma} = f \cdot \frac{P_{aroma,sample}}{P_{IS,sample}} \cdot \frac{m_{IS}}{m_{sample} \cdot \varphi_{DM}} \qquad [1]$$

$$f = \frac{P_{IS,reference}}{P_{aroma,reference}} \cdot \frac{m_{aroma,reference}}{m_{IS,reference}} \qquad [2]$$

In equations 1 and 2: C_{aroma} is the concentration of the aroma; f is the factor of proportionality of detector response; $P_{aroma,sample}$ is the peak area of aroma measured in the sample; $P_{IS,sample}$ is the peak area of internal standard measured in the sample; m_{IS} is the mass of internal standard added; m_{sample} is the mass of sample; φ_{DM} is the mass fraction of dry matter; $P_{IS,reference}$ is the peak area of the internal standard in the reference; $P_{aroma,reference}$ is the peak area of the aroma reference.

9. Results and discussion

9.1 Investigation of aroma components

The investigation of aroma profile was performed using solvent extraction followed by GC analyses as described above. The target components identified were esters (methyl hexanoate, methyl octanoate, ethyl hexanoate and ethyl octanoate) and fatty acids (hexanoic acid and octanoic acid). The results are shown in Table 3. It is seems that hexanoic acid and octanoic acid are the most abundant aroma compounds in *Vangueria infausta* while the methyl and ethyl esters respectively appear in relatively low amounts. We should note that, paradoxically, the aroma perceived from the fruit comes from ethyl hexanoate and ethyl octanoate even when they present low concentration. This fact can be understandable if we look at threshold values of these components. The fatty acids have highest threshold values (3000µ/L) against not more than 5µg/L for esters (Pino, 2006). Therefore the odor activity values exhibited by esters are higher than that one exhibited by fatty acids despite the high amount (Attaie, 2009; Guth, 1997).

Table 3. Concentration of potential aroma components calculated as function of dry matter and fresh weight.

Aroma Compound	Concentration (µg/g,DM)	(µg/g,fW)	Threshold µg/L	OAV*	OAV**
Hexanoic acid	5600	1800	3000 [b]	1,9	0.6
Octanoic acid	240	90	3000 [b]	0,08	0.03
Ethylhexanoate	44	14	1 [a, b, c]	44	14
Ethyloctanoate	13	4,2	5 [d]	2,6	0.84
Methylhexanoate	15	4,9	70 [b]	0,22	0.07
Methyloctanoate	12	3,7	200 [a, b]	0,06	0.019

The reference from which the threshold value has been taken is given in superscripted lower case letter: [a] - by Monteiro-Calderón et al. 2010; [b] - by Pino and Mesa, 2006; [c] - Takeoka, et al. 1995; [d] - by Gómez et al. 2012; Wu et al., 2011, Qian and Wang, 2005

* - Give as function of Dry matter

** - Give as function of fresh matter

9.1.1 Variation of aroma components between collection events

A set of experiments involving samples collected during four consecutive years (2010-2013) were performed to evaluate the variability of the aroma profile into the material. The experiments were conducted in three replicates for the sample belonging to each season and from each season-replicate; one extraction was performed, with three replicates for each. The target components were found in samples from all seasons (Table 4). In general the The variation of concentration is considerably small.

Table 4. Concentration (µg/g, DM) of the target aroma compounds isolated from *Vangueria infausta* over four consecutive seasons.

Aroma component	Season (harvest year) Concentration (µg/g, DM)				
	2010	2011	2012	2012a	2013
Hexanoic acid	4640	6000	5600	30700	4400
Octanoic acid	320	350	240	2000	300
Ethylhexanoate	48	43	44	440	44
Ethyloctanoate	24	20	13	230	20
Methylhexanoate	8	17	15	59	17
Methyloctanoate	9	9	12	66	11

One way ANOVA (Table 5) showed significant variation of the concentration between different seasons. The relative standard errs (rStdE) for ethyl hexanoate, ethyl octanoate and methyl hexanoate are rough of the same magnitude (14%) which shows a deviation within measured replicates. Losses during the extraction step and other uncontrolled factors during the analysis could contribute to this variation.

In all cases the calculated F-values are greater than F-critic which suggests the significance of the measurement. The relative standard errs within replicates for each season is in general small except for methylhexanoate which presents rSidE of 32%. The low rStdE verified for hexanoic acid and octanoic acid (13 and 25% respectively) could not fully represent efficiency of measurement but probably just because these components are in high amount and so maybe, the loss is not very expressive here.

It is believed that many factors such as climatic changes between seasons, rains or drought, fertility, soil conditions, differences of the trees and locality where the sample has been collected, and especially the variation in maturity of the fruits, could contribute to these discrepancies at least between seasons. Nonetheless, the major finding is that the variation between seasons is small.

Table 5. Analysis of Variance of concentration of target aroma components extracted from *Vangueria insfausta* from four consecutive seasons (2010-2013).

Arom Compound	*Source of Variation*	Avrag Conc. (2010-2013) µg/g,DM	*F-value*	*F-crit*	SDVT	Relative SDT Error (%)
Hexanoic acid	Between Groups (seasons)	10100	230	3,5	19200	
	Within Groups (repl. in each season)				1260	13
Octanoic acid	Between Groups (seasons)	620	70	3,5	1230	
	Within Groups (repl. in each season)				150	25
Ethyl hexanoate	Between Groups (seasons)	130	260	3,5	300	
	Within Groups (repl. in each season)				19	15
Ethyl octanoate	Between Groups (seasons)	61	520	3,5	160	
	Within Groups (repl. in each season)				7	12
Methyl hexanoate	Between Groups (seasons)	24	25	3,5	40	
	Within Groups (repl. in each season)				8	32
Methyl octanoate	Between Groups (seasons)	21	230	3,5	40	
	Within Groups (repl. in each season)				3	13

9.1.2 Sensory analysis

A tentative aroma description of *Vangueria infausta* was carried out by sniffing the fruit and comparing this with the smell of a reference compound. The panel was comprised of 10 people aged between 23-60 years who were staff members at the Department of Food Technology at Lund University, and who had previously, in some way, experienced the sensory evaluation of food and beverages using qualitative descriptive analysis. Table 6 summarizes the aroma description of the most pronounced volatile found in *Vangueria infausta.*

Roughly 90 % of the panelists concluded that the *Vangueria infausta* has a smell comparable to ethyl hexanoate and/or ethyl octanoate. Findings from the literature review show that these compounds have a strong similarity with the fruity smell of banana, apple, floral and fresh odor descriptions (Francis and Netwon, 2005, Qian and Wang, 2005, Gomez et al, 2010). This non-uniform description can be easily understandable if we take into account the difficulty and weakness in our ability to describe what we recognize, and to select the proper smell among numerous flavors released at the same time from the fruit. Another reason is that the total intensity is a sum of the individual OAVs of each component, regardless of the rest of the compounds presents in the sample (Laska and Hudson, 1992, Le Guen et al., 2000, Lopez et al., 2003).

Table 6. Smell description of aroma components identified in extracts from *Vangueria infausta*.

Fruit or Aroma compound	Odor description	
	From the panellist	From the literature
Vangueria infausta fruit	The smell resembles that of a banana. The odor is somewhat fruity, with a slightly vinous character, somewhat like green apple and pineapple that is characteristic for the African medlar.	Not found
Methyl hexanoate	The smell is fruity. It's like pear.	Ethereal fruity (pineapple-apple) [g].
Ethyl hexanoate	The smell resembles that of a banana. It's very fruity.	Fruity [a, c, d, e, g], banana [d, e, g h], green apple [b, c, d, g, h] strawberry [c], anise [c], brandy [d], apple peel [a], winey [g], wine [g], apple [g], pineapple notes [g], strong [g].
Methyl octanoate	The odor is somewhat sharp and fruity.	Strong, [g], winey-fruity [g], orange-like [g].
Ethyl octanoate	The smell is very close to floral character. It's somewhat fruity.	Fruity [a, b, d, e], floral [c, d, e , i] sweat [b], pineapple [c, h], pear [c, d], banana [d], brandy [d], fat [a], soapy [h].
Hexanoic acid	The smell is rancid or like cheese. It's like goats smell.	Cheesy [c, d, h], sweat [a, b, f], rancid [c, d, e], fatty [d].
Octanoic acid	Resembles milk or coconut liquid. It's somewhat rancid/cheese.	Rancid oily [h], sweat [a, b, f], cheesy [a, b, c, d], rancid [c, d], fruity [c, d], fatty [c], sour [e], goaty [e], fatty [c].

Superscripted lower case letter indicate the source in which the value has been taken*:* a-Francis et al. (2005), b-Gomez et al. (2012), c-Li-Hi et al. (2008), d-Wu et al. (2011), e-Qian et al. (2005), f-Ong, et al. (1998), g-Kaewtathip and Charoenrein (2012); h-Zea, et al. (2007); i-Munoz et al, (2007)

9.2 Drying of *Vangueria infausta*

In order to evaluate the aroma retention of *Vangueria infausta* during drying measurements of the volatile concentration during a controlled drying an experiment was performed. A sample of *Vangueria infausta* was dried in an isothermal convective dryer at 80 °C at a velocity of 3 m/s as described by Chiau, (Chiau, 2013).

Thirty-five batches of homogenized pulp (5 g on each) were packed into a mold, placed on a tray and then removed after 60, 120, 180, 240, 300, 360, and 420 minutes of drying. The temperature profile was controlled by using thermocouples (type K, 0.5 mm) connected to the hp Compaq S720 computer. Dry Matter (DM) and water activity (a_w) were measured after each drying time during the course of the drying process as described in paper II.

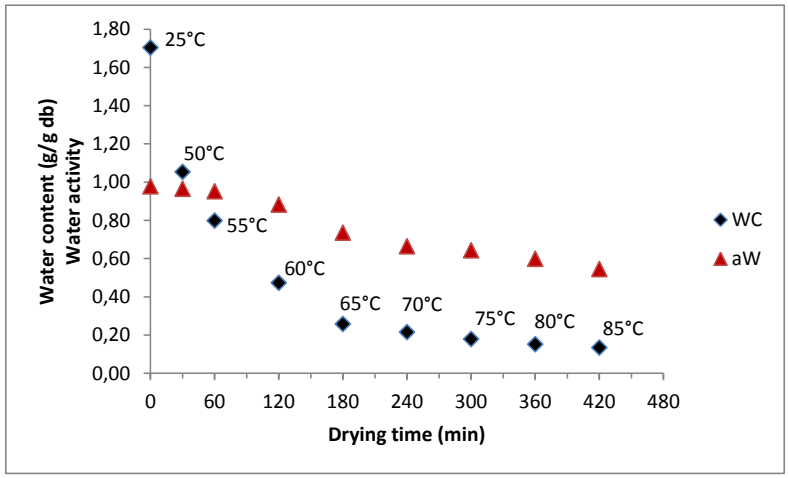
The decreasing water content and water activity was followed as a function of drying time during the convective drying of *Vangueria infausta*. Samples dried for 240-300 minutes give an average DM and water activity equal to 0.20±0.03 g/g, and dry basis equal to 0.65±0.01 respectively. The drying has to be continued for a sufficiently long time to reach humidity below a water activity of 0.70 as there is a high risk of growth of molds above that level (Tsotsas and Mujumdar, 2011, Jangam et al., 2008). However, at the same level of dryness the product tends to obtain serious hardness that makes direct consumption difficult (Chiau, et al., 2013). From the point of view of preservation of food, water activity in the range of 0.60-0.70 is assumed to be the most acceptable. (Paper II).

9.3 Aroma retention

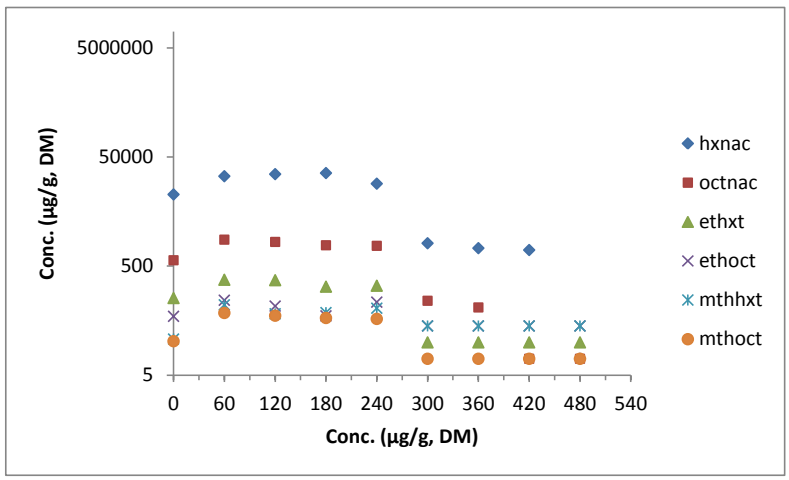
The results in figure 6 (A, B) tell us that the water is removed quickly during the first 180 min of drying followed by a slow drying period with a decreasing rate from 180 min to 420 min. Similar behavior is also observed in the water activity parameter (figure 6A). On the other hand, through the figure 6B it is possible to see that initially all aroma components (esters and fatty acids) are retained within the fruit matrix at least until 240 min of drying time (corresponding to water content of 0.20 g/g dry basis and a water activity of 0.65). At this time there is a dramatic release of aroma material. After 300 min only fatty acids remain within the fruit matrix. However the main outcome is that we can remove almost 85% of water without any loss of aroma compounds.. The experiment was repeated and the results were confirmed.

A possible reason of aroma loss could be the volatilization of volatiles due to the increasing sample temperature during the drying process (Coumans, 1994). However, as can be observed in Figure 6, the aroma loss is more or less independent of the volatility (e. g. the boiling point) of the individual aroma components and the temperature increase between 240 and 300 minutes is from 70°C to 75°C. Hence, general volatility seems an unlikely explanation to the sudden aroma loss after 240 minutes of drying.

Another possible explanation is that a sudden crystallization is taking place when the water content is around 10-15% leading to microstructural changes releasing entrapped volatile components (Roos, 1995, Salmon, et al., 1996). Microscopy observations were made (Paper II) but they could not clearly support a dramatic crystallization within the matrix as there was a dominating presence of crystalline cellulose and hemicellulose in the material making the observation non-conclusive. However our assumption that the loss of the target aroma compounds (esters) could be due the sugar crystallization phenomena is supported by the estimated phase boundary between the liquid state and the crystalline state for a complex sugar solution (Paper II)



A



B

Figure 6. Behavior of aroma components of the *Vangueria infausta* during convective oven draying set at 80°C, air velocity of 3 m/s: A - Relationship between water content on dry basis, a_w, temperature profile and drying time during drying of the *Vangueria infausta*. B - Concentration of aroma components during the drying. hxac –hexanoic acid; otac –octanoic acid; ethxt –ethyl hexanoate; etoct –ethyl octanoate; mthxt –methyl hexanoate and mtoct – methyl octanoate.

10. Conclusions

The extraction of aroma has to be effective and this demands a starting point of the work. The aroma components can be extracted using ethanol. Sugars tend to follow with the extract leading to the formation of degradation products during the analyses. Thus, the inclusion of a cleaning step removing all sugars is quite essential for obtaining a material that can be analyzed.

The volatile components found in pulp from *Vangueria infausta* is primarily: Hexanoic acid (30000-4000 µg/g, DM), octanoic acid (2000-200 µg/g, DM), ethyl hexanoate (400-40 µg/g, DM), ethyl octanoate (200-10 µg/g, DM), methyl hexanoate (60-8 µg/g, DM) and methyl octanoate (70-9 µg/g, DM). Based on the odor activity values it can be concluded that the odor of the fruit mainly originates from ethyl hexanoate and ethyl octanoate. Fruity, sweet, floral and slightly fatty are the attributes that most mark the aroma profile of the studied fruit.

The principal aroma components of pulp from *Vangueria infausta* are well preserved during the initial phase of drying (down to about a relative water activity 0.65). However, the aroma components are lost after a more extensive drying. A possible explanation of the volatilization is sugar crystallization that is to occur below a relative humidity of around 0.70 during the drying process.

The results highlighted, to a certain extent, the potential of *Vangueria infausta* as a wild fruit to be included in industrial food products. However, more studies are needed in order to improve the retention of volatiles during drying. Drying and encapsulation of these volatiles could help to sustainably utilize the diversity of wild fruits growing in Mozambique.

11. Further perspectives

The next step is to investigate the role of the particular structure of the fruit matrix for aroma retention. For this purpose, a possible model system may involve pectin and micro-crystalline cellulose crystalline. Once a better understanding of aroma retention has been obtained, we can accomplish a better explanation of the background.

We believe that the encapsulation of these aromas can bring added value to the great diversity of wild fruits as well as to extending the evaluation and assessment of fruits not only in gastronomy but also for the design of new lines of product formulations such as yogurts, powdered juice and infant foods, among others.

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Paper I

Identification of volatile components isolated from *Vangueria infausta* (African medlar) by using GC-MS

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Abstract

Volatile compounds isolated from the African medlar *(Vangueria infausta* L.) were identified by Gas Chromatography and Gas Chromatography-Mass Spectrometry. The volatile components identified in extract were: hexanoic acid (6000 µg/g, DM*)*, octanoic acid (240 µg/g, DM), methyl hexanoate (15 µg/g, DM), ethyl hexanoate (44 µg/g, DM), methyl octanoate (12 µg/g, DM) and ethyl octanoate (13 µg/g, DM). The principal aroma from the *Vangueria infausta* fruit originates from ethyl hexanoate and ethyl octanoate with a minor contribution from methyl hexanoate and methyl octanoate.

Keywords

Vangueria infausta; volatile components; GC-MS; aroma profile.

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1. **Introduction**

Fruits are an essential part of a healthy and culinary appetizing diet. They are considered to be rich sources of essential dietary micronutrients, and they have also been recognized as important sources of phytochemicals as mentioned by Asgari et al. (1) and Perez-Silva (2). The fruit part of the diet of southern Africa is dominated by common farmed fruits, but a wide range of more or less wild indigenous fruits is also consumed. A few studies relating to nutritional aspects of indigenous fruits of Mozambique (for example *Vangueria infausta, Landolpia kirki, Andonsonia digitata, Ziziphus mauritiana, Strychnos spinosa, Salacia kraussi, Anacardium accidentale*) have shown that most of these fruits have high levels of micronutrients like minerals and vitamins as described by Styger et al. (3), Amorteifio et al. (4), Magaia et al. (5) and Magdi et al. (6).

This study focuses on the fruit of the African medlar (*Vangueria infausta* L., family *Rubiaceae).* In Mozambique, *Vangueria infausta* is commonly called maphilwa (Ronga language) or wild medlar (South African English) and African medlar is used as english name in easern Africa. It is brown-orange when ripe and has an orange parenchyma tissue. The fruit is about 2-5 cm in diameter and contains three to five seeds. The fresh fruit is sweet, sour, somewhat bitter and slightly astringent, and the aroma remains of the common medlar (*Mesphilus sp.*) or the wild apple *(Malus sp*.). The pulp can be eaten fresh or cooked. The pulp is used to prepare juice, jam, marmalade and puddings by adding water and sugar, or cooked to make a kind of porridge. The fresh fruit can also be dried in the sun and then stored for long time. In the southern part of Africa, the fruit is usually harvested from February to April.

Fruits in general contain volatile compounds as described by Pino et al. (7), Monteiro-–Calderón et al. (8) and Liceraldello, et al. (9). The volatile chemicals present in natural leaves, flowers and fruits have been widely used in aromatherapy since ancient times, suggesting that they impart some beneficial health effect in addition to their pleasant oduor. This helps to add value in the evaluation and assessment of fruits, and has played an important role in gastronomy since ancient times (10). However, there have been few studies about the identification and quantification of aroma components in *Vangueria infausta.* Pino and co-workers (7) characterized the volatile components of the closely related *Vangueria madagascariensis*. They found 62 substances, including 2 and 3 furfural (15µg/g), hexadecanoic acid and tetradecanoic acid around 5 µg/g, shorter fatty acids (hexanoic, heptanoic and octanoic acids, around 6 µg/g), fatty acid methyl and ethyl esters (methyl hexanoate 4µg/g), other aldehydes (benzaldehyde, phenylacetaldehyde), alcohols (butanol, furfurylalcohol, etc, 2µg/g) and terpenoids (limonene 2µg/g). The aim of this work was to identify the aroma profile of *Vangueria infausta* and thereby determine the chemical components responsible for the fruitiness.

2. Material and methods

2.1. Chemicals

The solvents (ethanol, pentane, diethyl ether) and the reference compounds (hexanoic acid, octanoic acid, methyl hexanoate, ethyl hexanoate, methyl octanoate and ethyl octanoate) were purchased from VWR International (Radnor, Pennsylvania, USA). Geraniol was used as internal standard (IS). IS was of chromatographic purity, acquired from Merck, Darmstadt, Germany. All chemical solvents were of analytical grade while standards were reagents of technical grade. Distilled and deionised water was obtained from a Milli-Q ionic exchange and carbon filter apparatus (Millipore, Bedford, MA, SA). The gases for GC (helium, nitrogen and oxygen) were of 99.9% purity (from L'Air Liquide S.A, Paris, France).

2.2 Raw material

Ripe fruits of Vangueria infausta were collected in Bobole, a rural village located approximately 30 km north of Maputo city in Mozambique during the first week of March 2012. The fruits were collected from different trees located in the same area within the radio of approximately fifty meters. The fruit were kept in plastic basket and transported to lab at Chemistry Department (Eduardo Mondlane University). Only fruits without dameges at the surface and of a fullgrown size, approximately 4 cm in diameter, were selected. The fruits were washed with tap water andwere packed in vacuum bags (15-20 units in each bag), sealed and frozen at minus 20°C for two weeks. Then, the fruits were transported to Lund University (Sweden) using a thermo bag in order to keep the sample frozen during the transport.

2.3 Sample preparation

Before the experiment, 2 packed vacuum bags (about 35 fruits in total) were thawed at room temperature and then boiled in water during 10 min to inactivate the enzymes like lipoxygenase (LOX), hydroperoxidelya se (HPL) and alcohol dehydrogenase (ADH). This to suppress production of C_6 volatiles as observed by Bai et al. (11). The fruits were allowed to cool, peeled using a clean knife and the seeds were manually separated from the pulp. The pulp were mashed and homogenised by stirring with a clean spoon. The pulp was packed in vacuum bags.

3. Extraction of volatiles

The volatiles were isolated using liquid/liquid extraction. The selection of extraction solvents was to release volatiles entrapped in the structure by using semipolar solvent with good abilities to penetrate the tissue. Ethanol (96%) was chosen for this purpose.

40 g of fruit homogenate (moisture content equal to 68%, 33.4 °Brix) was initially mixed with 150 ml of ethanol (with geraniol, 2.3 $.10^{-3}$ mol/L, as internal standard) and stirred during forty-eight hours to produce a suspension of fruit particles in a aqueous-ethanol liquid phase. Similar procedure was carried out by Hattab et al. and Sides (12, 13). The suspension was centrifuged at 3000 rpm, 15 min, 18 °C on an AllegraX-15R (Beckman Coulter Inc., Brea, Californ ia, USA). The supernatant was used as raw extract. 20 ml of raw extract was cleaned from polar components (e. g. saccharides) using a hydrophobic column containing a bonded hydrophobic phase of desisopropyl atrazine 10% (Bakerbond $C_{18,}$ Capitol Scientific Inc., Austin, Texas, USA). The extraction liquid was diluted with water (ratio 3:1) to ensure high affinity of nonpolar components to the carrier phase. The diluted extraction liquid was allowed to pass through the column followed by a washing step of 10 ml water. Thereafter, the entrapped material in the column was eluted with 25 ml of a (1:1) mixture of pentane and diethyl ether. The experiment was conducted in triplicates.

4. GC-analyses

The aroma extract was analysed using Gas Chromatography (GC). The GC apparatus was Agilent Technologies 6890N (G1530), Network GC system, serial US10322054, purchased from J&W Scientific, USA. The equipment had a hydrophobic DB-225 column (length 30,0 m, diameter 250.0 µm; film thickness 0.25 µm), containing hydrophobic stationary phase of cyanopropylphenyl-dietylpolysiloxane (50%). The oven temperature program started from an initial temperature of 50°C, was kept constant for 3 min, and then increased at a rate of 3°C/min to 200°C, over 54 min. The injector and detector temperatures were 250°C. Helium was used as carrier gas, flow rate of 0.6 ml/min, pressure 55.2 kPa. Inlet split ratio was 20:1 and split flow of 12 ml/min. Hydrogen flow at 30 ml/min. The injector had a syringe size of 10µl, volume sample of 2.0 µl, was injected three times, and n-hexane was used to wash five times.

The GC-MS analysis carried out on an coupled gas chromatography-mass spectrometry, Agilent (Palo Alto CA, USA) 5972 mass selective detector, together with an Agilent 6890 GC equipped with a HP-5MS or Innowax capillary column (30 m x 0.25 mm i.d., SGE, Austin, TX, USA). The oven temperature was programmed from 50°C, held constant for 3 min, increased by 5°C/min to 230°C, then held constant for 10 min. The MS data was compared with mass spectra of fragment pattern from the MS databank from NIST (National Institute of Standards and Technology) library. This chromatographic method has been applied in previous works Glasl et al. (14), Janes et al. (15), Reineccius (16) and Kaewtathip et al. (17). The amount of each aroma compound was calculated in relation to the dry mater (DM) of the sample, taking into account the signal intensities of the reference

compounds. The retention index (RI) of each target compound is calculated using n-paraffin references (C_{12}-C_{24}) on a PH-88 column (30 m x 0.25 mm i.d., and 0.20 µm film thickness; J&W Scientific, Agilent Technologies).

The amount of each aroma compound was calculated in relation to the dry mater (DM) of the sample, taking into account the signal intensities of the reference compounds. Equations 1 and 2 were used for the aroma concentration calculation:

$$C_{aroma} = f \cdot \frac{P_{aroma,sample}}{P_{IS,sample}} \cdot \frac{m_{IS}}{m_{sample} \cdot \varphi_{DM}} \quad (1)$$

$$f = \frac{P_{IS,reference}}{P_{aroma,reference}} \cdot \frac{m_{aroma,reference}}{m_{IS,reference}} \quad (2)$$

C_{aroma} is the concentration of the aroma; f is the factor of proportionality; $P_{aroma,sample}$ is the peak area of aroma measured in the sample; $P_{IS,sample}$ is the peak area of internal standard measured in the sample; m_{IS} is the mass of internal standard added; m_{sample} is the mass of sample; φ_{DM} is the fraction of dry matter; $P_{IS,reference}$ is the peak area of the internal standard in the reference; $P_{aroma,reference}$ is the peak area of the aroma reference.

5. Results and discussion

The GC analyses of the raw extract of *Vangueria infausta* produced results dominated by a wide range of peaks, of which more than one was identified as different furfurals by MS as mentioned by Raice et al. (18), in agreement with the study of aroma profile of *Vangueria madagascariensis* by Pino et al. (7). However, further experiments in which a comparable extract of an aqueous sugar solution (glucose, fructose and sucrose) was injected showed that the furfurals were caused by the presence of the sugars in the injected extraction phase and that the different furfurals were probably decomposition products when the sample were injected into the column in the oven. Similar degradation has been observed by Cheetham (19).

A cleaning procedure using a hydrophobic column as described in the method section was introduced to eliminate the sugars from the extract. The yield of the internal standard was measured to about 80% of the complete extraction and cleaning procedure.

Figure 1 shows a GC chromatogram of the extract after the cleaning procedure. The number as well as intensity of the peaks is much reduced, and none of them represent typical saccharide decomposition products such as furfurals**.**

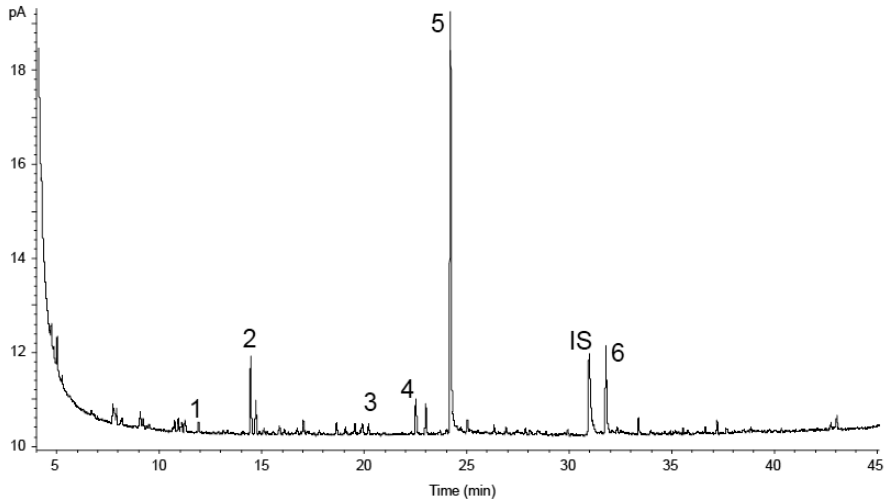

Figure 1. FID chromatogram of *Vangueria infausta* as obtained using a DB-225 column (30m x 0.25 mm x 0,25µm film thickness): 1-methylhexanoate (Rt=12.02 min), 2-ethylhexanoate (Rt=14.55 min), 3-methyloctanoate (Rt=20.29 min), 4- ethyloctanoate (22.59 min), 5-hexanoic acid (Rt=24.29 min), 6-octanoic acid (Rt=31.86 min), 7-geraniol (IS) (Rt=31.03 min).

The peaks obtained were reanalysed for tentative identification using GC-MS. Two different columns were used to control eventual co-elution. One column was a high polar Innowax and one a non-polar HP-5. The GC results from these two columns gave different orders between peaks and some of the peaks fused together when the polar column were used. As the use of polar column may result in poor resolution of fatty acids, another experiment with methanolysis conversion was performed on a polar HP-5 column. The reference materials of the target aromas were used in order to verify the identification. All tentative identifications were confirmed with reference compounds (MS spectra and retention time). The mass spectra pattern is shown on Figure 2.

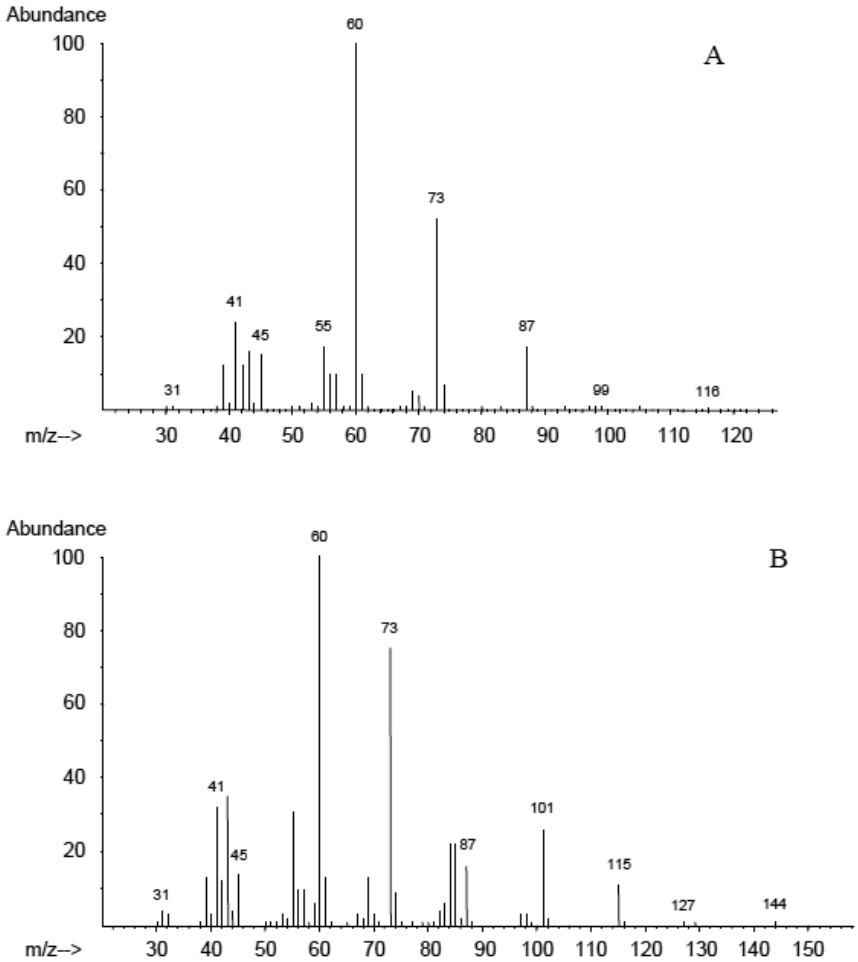

Figure 2. Mass spectra of the most abundant compound: Hexanoic acid (A) and octanoic acid (B) identified from the *Vangueria infausta* extract.

The mass spectrum of hexanoic acid showed intensive rearrangement fragments at *m*/*z* 60, which, in combination with ion at *m*/*z* 73, suggested an aliphatic carboxylic acid structure. The tentative molecular ion at *m*/*z* 116 and 99 $(M\text{-}OH)^+$ in the spectrum indicated a saturated C_6 acid, which was finally confirmed as n-hexanoic acid by comparing the retention times and mass spectra with the reference compound under both polar (INNOWax) and non-polar (HP-5) columns. Similar fragmentation pattern was observed in the spectrum of the second most abundant homolog octanoic acid in which a tentative molecular ion at *m*/*z* 144 implied two

CH_2 units longer than hexanoic acid and the compound was finally identified as saturated n-octanoic acid.

The methyl hexanoate and methyl octanoate showed typical mass spectra of the methyl esters of corresponding hexanoic and octanoic acid, characterized by the combination of the McLafferty rearrangement ions at *m*/*z* 74 and 87, as well as the $(M\text{-}OCH_3)^+$ fragment at *m*/*z* 99 and 127, and the molecular ion at *m*/*z* 130 and 158, respectively. Whereas components ethyl hexanoate and ethyl octanoate presented the mass spectra of ethyl esters of hexanoic and octanoic acid, characterized by the ions pair at *m*/*z* 88 and 101, the $(M\text{-}OCH_2CH_3)^+$ fragment at *m*/*z* 99 and 127, and the molecular ion at *m*/*z* 144 and 172, respectively. All these esters were further confirmed by the synthetic references.

The final outcome of the identification is shown in Figure (1). Disulphide, hydrocarbon (octane, undecane, tridecane), BHT (butylated hydroxytoluene) and DTBP (di-tert-butylporoxide) were also found. BHT is included in the diethyl ether as an antioxidant to control peroxide formation. The DTBP probably originates from the polyethylene bags (used as initiators for the polymerisation reaction). The sulphide is probably also originates from the polymer synthesis. The remaining substances were considered potential aroma. Table 1 shows the retention index (RI) of each target compound calculated using n-paraffin references (C_{12}-C_{24}). The small peaks of impurities and not identified material are well separated and do not bring significant interference on the determination, we neglected them.

Table 1. Retention Index of most potent aroma compounds found in *Vangueria infausta*

Aroma Compound	Rt (min)	RI
Methyl hexanoate	6,743	1295,2
Ettyl hexanoate	7,686	1341,2
Methyl octanoate	10,724	1489
Etthyl octanoate	11,659	1536,1
Hexanoic acid	17,376	1850,6
Octanoic acid	23,796	2273,1

The peaks were quantified using the intensity relative to the intensity of IS peak and compared with the ratio of intensity between reference materials and IS. The major constituents were hexanoic acid (5600µg/g, DM), octanoic acid (240 µg/g, DM), methyl hexanoate (15 µg/g, DM), ethyl hexanoate (44 µg/g, DM), methyl octanoate (12 µg/g, DM) and ethyl octanoate (13 µg/g, DM). The description of aroma and the quantitative data is listed in Table 2. Most of the identified components were reported in the previous study on *Vangueria madagascariensis* by Pino et al. (7). For example hexanoic acid, octanoic acid and their corresponding methyl esters have been found. However, Pino and co-workers did not find the ethyl esters identified in this study.

Table 2. Description of odour of volatile components identified in extracts from *Vangueria Infausta.*

Compound	Concentration (µg/g, DM)	Concentration (µg/g, fW)	Odour threshold (µg/l)	OAV	Odour Description (in this investigation)
Hexanoic acid	5600	1800	3000 [b]	5,9E+02	The odour is somewhat rancid or cheesy. Resembles of the smell of goats.
Octanoic acid	240	90	3000 [b]	2,5E+01	The odour is somewhat rancid or cheesy, but milder than hexanoic acid. Resembles of coconut milk or liquid.
Ethyl hexanoate	44,3	14,2	1,0 [a, b, c]	1,4E+04	The odour is fruity. Resembles of banana.
Ethyl octanoate	13,2	4,2	5 [d]	8,5E+02	The odour is somewhat fruity, with a slightly wine character. Resembles of the African medlar, *Vangueria infausta*.
Methyl hexanoate	15,2	4,9	70 [b]	6,9E+01	The odour is sharp and fruity. Somewhat pear like.
Methyl octanoate	12	3,7	200 [a, b]	1,9E+01	The odour is somewhat sharp and fruity.

The reference from which the threshold value has been taken is given in superscripted lower case letter: a – by Monteiro-Calderón et al. 2010 (8);
b - by Pino and Mesa, 2006 (20); **c** - Takeoka, et al. 1995 (31); d - by Gómez et al. 2012 (32).

The impact of an aroma for the perception of the fruit is a concentration issue. The odour activity was estimated to evaluate the impact of the individual compounds for the actual aroma profile of the fruit. OAV is the ratio between the concentration and the threshold value of the aroma compound as described by Pino et al. (20), Guadagni et al. (20) and Wright et al. (22). According to Cheetham (19) the probability of a compound's odour being detected is expected to be greater with increasing OAV. In this study the OAV of each compound was estimated from the threshold values measured in aqueous solutions found in literature.

The results show that the ethyl and methyl esters, in particular ethyl hexanoate, are expected to be the most powerful contributors of aroma in the fruits despite the low concentration of these esters. This is because the threshold values are comparatively low and the OVA consequently high. The short-chained fatty acids have a comparable weak odour as observed by Elss et al. (26) and Attaie et al. (27) and this explain the low OAV exhibited by hexanoic and octanoic acids despite the high concentration.

To verify the evaluation of the contribution of each component to the flavour of the *Vangueria infausta* fruits, the flavour of each individual component was compared. The

results were found to be in somewhat agreement with similar descriptions in previous studies by Kaewtathip et al. (17), Gomez et al. (28), Li et al. (29) and Zea et al. (30). The evaluation was made by sniffing freshly-cut pieces of the fruit as well as the pure compounds. The aroma presented with this fruit predominantly originates from esters, which gives a characteristic fruity flavour with a slight element of banana, green apple and pineapple that is characteristic for the *Vangueria infausta* (African medlar).

6. Conclusion

The principal aroma in the *Vangueria infausta* fruit is originates from ethyl hexanoate and ethyl octanoate with a minor contribution from methyl hexanoate and methyl octanoate.

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Paper II

Characterization of aroma loss after convective air-drying of *Vangueria infausta L.* (African medler) *by* using GC

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Abstract The loss of aroma components after drying of the fruit of *Vangueria infausta L.* was studied by means of convective air-drying and GC techniques. The samples were dried at 80°C, air flow 3 m/s at different drying time in the range of 60 up to 420 min. Aroma composition, dry matter and water activity were measured for each sample along of drying time. The study shows that the target aroma components (hexanoic acid, methyl hexanoate, methyl octanoate, octanoic acid, ethyl hexanoate, ethyl octanoate) that are present in fresh fruit sample are retained in the matrix at least until 240 min of drying. Sample dried at 300 min showed decreased amount of volatiles and practically no aroma where found after 420 min. The result is explained as a consequence of crystallization of sugars that occurs at water activities below 0.8 and that this may be the reason for the losses of aroma.

Keyword: Vangueria infausta, aroma, water activity, crystallization.

1. Introduction

Drying is an ancient technique used for preservation of fruits. A critical aspect is to what extent desirable properties of the fruit is maintained during the processing. Among these properties one of the most challenging quality attributes is the aroma [1,2]. Drying a process that is particularly likely to cause quality loss [2]. Several types of drying like spray drying and freeze drying has been used to study the aroma retention using model systems consisting of aqueous carbohydrate solutions containing small amount of aroma component [3-5]. In several studies, the mass transfer in the drying material matrix is described as a diffusion process. A particular issue is the role of the matrix forming the continuous structure of the dried food. The continuous structure in most dried fruits is mainly composed of different carbohydrates. The carbohydrates may be present in crystalline or in a glassy state and it has been observed that the flavor retention is much higher in the presence of a continuous glassy structure than if the continuous structure is polycrystalline [6]. It has been suggested that low mobility in the glassy state reduce chemical reactions as well as mass transfer [7]. The achieved water activity by the drying process plays an important role to the physical state of the encapsulation matrix [8-10].

Vangueria infausta (African medlar) is an underutilized wild fruit growing in southern Africa. A commercial use demands rapid and effective preservation that thereby motivate studies of the drying properties. The fruit has an attractive flavor as being rich in short fatty acids and ethyl and methyl esters of the same acids [11, 12]. The fruit also have clear nutritional benefits as a source of vitamins and soluble fibers [13]. The objective of this study is to monitor the retention of volatailes during the drying process and to describe eventual loss of aroma retention.

2. Material and Methods

Ripe fruits of the *Vangueria infausta* were collected in Bobole, Maputo, Mozambique during the harvest season of 2012. Mature fruits, free defects, were selected, thereafter washed, sealed in polyethylene plastics bags and frozen at minus 20°C. Before use, the fruit were thawed at room temperature during 24 hours. After thawing the fruit was blanched in boiling water for 5 minutes to eliminate enzymatic activity. Peels and seeds were manually separated from the pulp using a knife. The fruit pieces were disintegrated and homogenized by using a domestic puree sieve. Forty samples with 5 g in each were placed in a mold and left to dry on an isothermal convective oven at 80 °C, 3 m/s.

2.1 Chemicals

The solvents (ethanol, pentane, diethyl ether), the reference compounds (hexanoic acid, octanoic acid, methyl hexanoate, ethyl hexanoate, methyl octanoate and ethyl octanoate) were purchased from VWR International (Radnor, Pennsylvania, USA). The internal standard (geraniol), were also of chromatographic purity, acquired from Merck (New Jersey, USA).

All chemical solvents were of analytical grade while standards were of reagents or technical grade. Deionized and purified water was obtained from a Milli-Q ionic exchange and carbon filter apparatus (Millipore, Bedford, MA, .SA). The gases for GC (helium, nitrogen and oxygen) were of 99.9% purity (from L'Air Liquide S.A, Paris, France).

3. Sample preparation

3.1 Drying of *Vangueria infausta* pulp

40 samples of *Vangueria infausta* pulp with 5 grams of pulp in each, were filled into spherical molds (5 mm thickness, 28 mm in diameter) purposely designed for this experiment and were dried in an convective oven set at 80°C, 3 m/s. The drying procedure has been described in [14]. The experiment was distributed as follows: Thirty-five samples were placed on a tray and removed after 60, 120, 180, 240, 300, 360, and 420 minutes of drying. The temperature profile was followed using (type K, 0.5 mm) thermocouples connected to a HP Compaq S720 computer. Water content and water activity (a_w) were monitored after each drying time along the drying process. The water content was obtained using a vacuum oven (Forma Scientific, Marietta, OH, USA), at 70 °C for 24 hours. Each sample was weighed on a Mettler AE160 analytical balance. The water activity (a_w) was measured at 20 °C using AquaLab water activity meter S3TE, supplied by Decan Devices, Inc (Pullman, Washington, USA) calibrated using saturated salt solutions (NaCl: a_w = 0.760 M and KCl: a_w = 0.984) (from Decagon Devices, Inc, Pullman, Washington, USA). The soluble solid was determined at 20 °C using a HI9681 digital sucrose refractometer with an accuracy of ±0.2% Brix (purchased from Woonsocket, Rhode Island, USA).

3.2 Extraction of aroma from the dried Vangueria infausta pulp

The extraction and GC analysis follows the previously described method [15].

Dried sample of *Vangueria infausta* pulp was rehydrated in 20 mL of distilled water and left to repose overnight before extraction. 40 mL of ethanol was added to the fresh or rehydrated dried samples and then stirred for 24 hours to allow for complete extraction. An internal standard (geraniol) was included with the ethanol. The obtained suspension of particles in a aqueous ethanol solution phase was centrifuged at 3000 rpm, 15 min, 18 °C on an AllegraX-15R, from Beckman Coulter Inc., Brea, California, USA. The supernatant was used as raw extract and was divided into three replicates of 10 ml each. The raw extract was diluted with 30 ml of water and was cleaned from polar components (e. g. saccharides) using a hydrophobic column. The hydrophobic column had a bonded hydrophobic phase of desisopropyl atrazine 10% (Bakerbond speTM Octadecyl (C18) from Capitol Scientific Inc., Austin, Texas, USA). [16].

The volatailes were recovered from the column by washing with a non-polar solvent mixture (pentane and diethyl ether) forming a uniform liquid phase with good solubilizing capacity for

non-polar volatiles [17]. The yield of the internal standard was measured and was found to be in range of 80%.

3.2.1 GC and GC-MS analysis

The aroma extract was analyzed using Gas Chromatography (GC). The GC apparatus was of model 6890N (G1530) from Agilent Technologies, Network GC system, serial US10322054, purchased from J&W Scientific, USA. A hydrophobic DB-225 column (length 30.0 m, diameter 250.0 µm; film thickness 0.25 µm), containing hydrophobic stationary phase of cyanopropylphenyl-dietylpolysiloxane (50%) was used. The oven temperature program started from an initial temperature of 50°C, was kept constant for 3 min, and then increased at a rate of 3°C /min to 200°C, over 54 min. The injector and detector temperatures were 250^{0}C. Helium was used as carrier gas at a flow rate of 0.6 ml/min, pressure 55.2 kPa, and split ratio of 20:1 flowing at 12 mL/min. Hydrogen flow at 30 ml/min. The injector had a syringe size of 10.0µl and a sample volume of 2.0 µl. The samples were injected with three repeated strokes and the syringe was rinsed five times with n-hexane between the samples.

The peaks were preliminary identified using GC-MS followed by a confirmation using reference standards as described by Raice [15]**.** An example of a GC chromatogram is shown in Figure 1.

The amount of each aroma compound was calculated in relative to the dry mater (DM) of the sample by relating the peak area of the substance to the peak area of the reference compounds. Equations 1 and 2 were used for the aroma concentration calculation:

$$C_{aroma} = f \cdot \frac{P_{aroma,sample}}{P_{IS,sample}} \cdot \frac{m_{IS}}{m_{sample} \cdot \varphi_{DM}} \qquad (1)$$

$$f = \frac{P_{IS,reference}}{P_{aroma,reference}} \cdot \frac{m_{aroma,reference}}{m_{IS,reference}} \qquad (2)$$

C_{aroma} is the concentration of the aroma; f is the factor of proportionality; $P_{aroma,sample}$ is the peak area of aroma measured in the sample; $P_{IS,sample}$ is the peak area of internal standard measured in the sample; m_{IS} is the mass (g) of internal standard added; m_{sample} is the mass of sample; φ_{DM} is the mass fraction of dry matter; $P_{IS,reference}$ is the peak area of the internal standard in the reference; $P_{aroma,reference}$ is the peak area of the aroma reference [18].

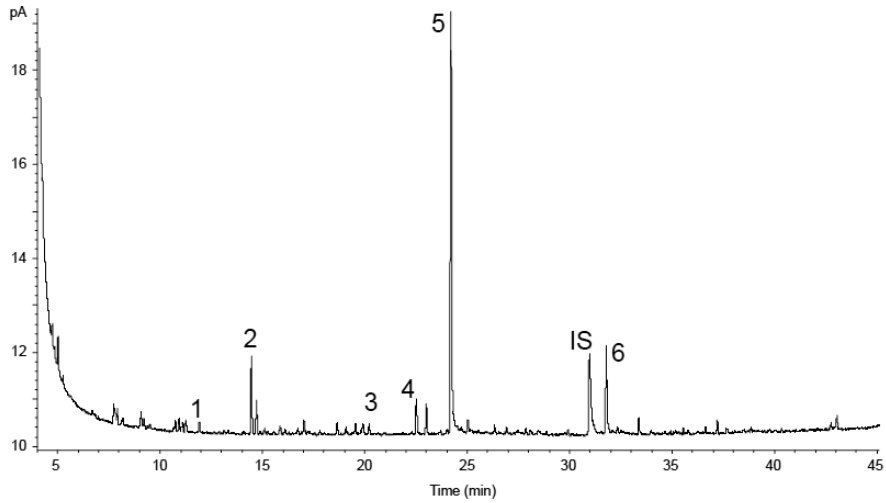

Figure 1. FID chromatogram for *V.infausta* sample as obtained using a DB-225 column: 1-methylhexanoate (Rt=11.91 min), 2-ethylhexanoate (Rt=14.45 min), 3-methyloctanoate (Rt=20.21 min), 4- ethyloctanoate (22.58 min), 5-hexanoic acid (Rt=24.21 min), 6-octanoic acid (Rt=31.79 min), 7-geraniol (IS) (Rt=30.95 min).

3.3 Microstructural observations.

Microstructural observations were made in a microscope equipped with a polarizing device and hot stage device. Samples were removed from the dryer (during a separate drying event) preserving the temperature (+2 -- -10 °C relative to the drying temperature) during the transfer to the hot stage of the microscope. The sample was disintegrated using a spatula and tweezers and immersed into coconut oil at the microscopic slide. The observations were performed within 10 minutes after the sample was removed from the dryer.

4. Statistical analysis

All analyses were conducted in triplicate for extraction. The results reported are the average of these three replicates. The statistical analyses were carried out using the Microsoft Office Excel 2007. Analysis of variance (ANOVA) was type general linear model (one way) procedure.

5. Results

5.1 Drying

The drying of *Vangueria infausta* pulp was performed at 80°C, 3m/s. Figure 2 shows the decreasing water content, water activity and dry matter as function of drying time during the convective drying. The samples display constant drying rate during the first hour followed by a decaying drying rate. Samples dried for 240-300 minutes gives in average water content and water activity equal to 0.20±0.03 g/g, dry basis and 0.65±0.01 respectively. From the point of view of preservation of food, water activity in range of 0.70-0.60 is assumed to be acceptable [19-21].

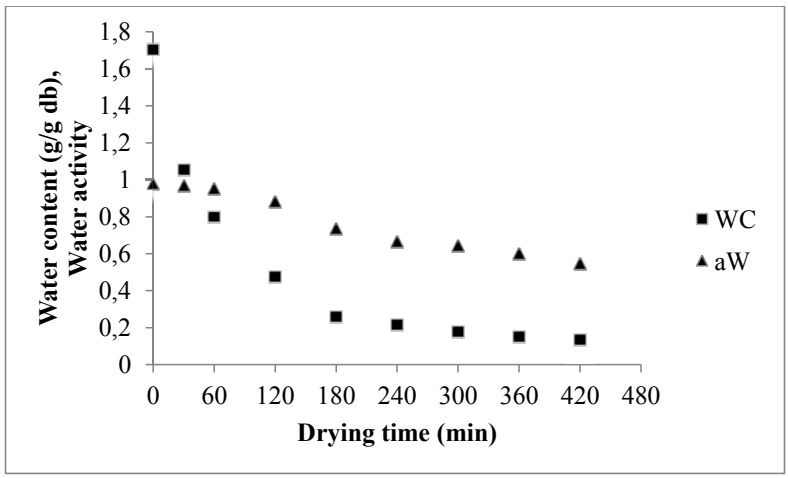

Figure 2. Relationship between water content on dry basis, a_w and drying time during a convective air-drying of *Vangueria infausta* at 80°C, 3 m/s.

5.2 Aroma analysis

The investigation of aroma profiles was performed using solvent extraction followed by GC analyses. The target components identified were esters (methyl hexanoate, methyl octanoate, ethyl hexanoate and ethyl octanoate) and acids (hexanoic acid and octanoic acid). The results are shown in Table 1. We can note that the fresh fruit is characterized by the fruity esters while the dried fruit is dominated by the free fatty acids after 300 minutes. The dramatic change during the drying is illustrated in Figure 3. The graphic shows that the fruit aromas are well retained during the first part of the drying. We are actually able to remove 90% of the water without loss of fruit esters. However, a dramatic change in the concentration of fruit esters is observed after about 240 minutes of drying. This loss of aroma retention in the fruit can be considered a major loss of quality and it occurs just when we have reached the target level in water activity (a_w=0.70-0.60) to obtain microbiological stability and is thereby a major challenge for the development of high quality dried fruit products of *Vangueria infausta*.

Microscopic observations were made to evaluate eventual changes in microstructural properties. From the images in Figure 4 it is possible to observe that the morphology drastically changes during the initial drying process. The cells changes from rounded into shrunk and wrinkled structures within the first hour. Intense birefringence was observed during the complete drying process due to the presence of crystalline fiber structures (initially) and later by the presence of both fiber structures and saccharide crystals. However, it was not possible to clearly identify the critical drying time for the crystallization of the saccharides in the fruit.

Table 1. Concentration (µg/g, DM) of aroma components isolated from *Vangueria infausta* after isothermal convective drying up to 420 min.

DM	Conc.(µg/g, DM)					
Drying Time(min)	Hexanoic Acid	Octanoic Acid	Ethyl hexanoate	Ethyl octanoate	Methyl hexanoate	Methyl octanoate
0	10300	640	130	60	23	21
60	22200	1500	280	118	98	69
120	24200	1400	276	92	67	62
180	25400	1200	210	61	70	56
240	16200	1177	220	109	85	54
300	1300	1200	< 20*	< 40	< 40	< 10
360	1060	87	< 20	< 40	< 40	< 10
420	986	< 10	< 20	< 40	< 40	< 10
480	< 10	< 10	< 20	< 40	< 40	< 10

<…* indicate the detection limit of the component

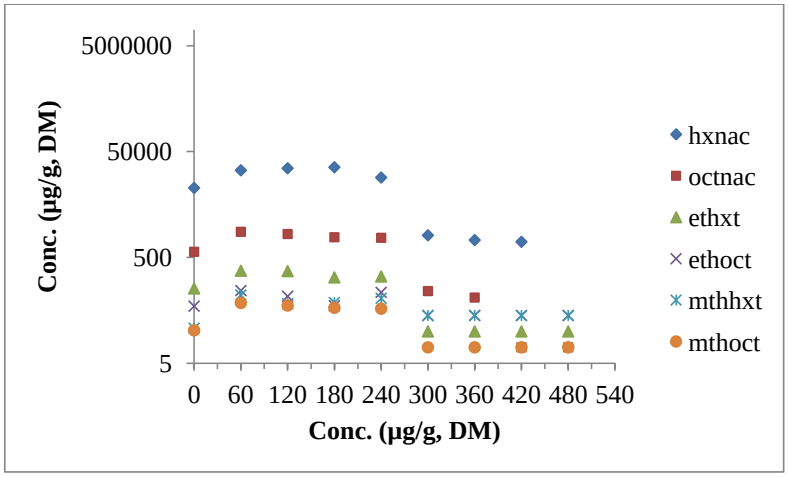

Figure 3. The aroma retention in fruit pulp from *Vangueria infausta* during a drying process. The sample was dried using an isothermal convective oven set at 80°C, air velocity of 3 m/s: hxnac –hexanoic acid; octnac –octanoic acid; ethxt –ethyl hexanoate; ethoct –ethyl octanoate; mthhxt –methyl hexnoate and mthoct –methyl octanoate.

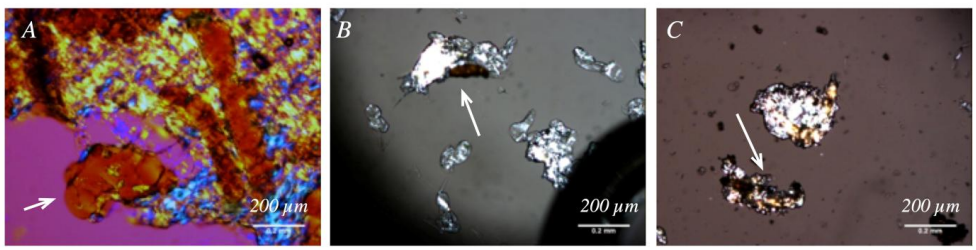

Figure 4. Changes in microstructure during drying of *Vangueria infausta*. The photos are taken using a 5x objective and polarizing light. The temperature is kept close to the sample temperature of the sample. A. Sample before drying. B. After 60 minutes. C. After 360 minutes. The arrows point on visible cells showing the shrinking with drying.

6. Discussion

Loss of aroma during drying may be caused by thermal degradation or volatilization [5]. Because the low drying temperature (80°C) and the late occurrence in the process, the loss of aroma components in this experiment, most likely, cannot be attributed to thermal degradation reactions. Therefore the most tangible explanation could be loss due to volatilization of the entrapped fruit esters. From Figure 3 it is clear that this is a sudden event, most likely due to a rapid change in the microstructure.

A possible process is a rapid crystallization of sugars also present in the fruit. Crystallization would squeeze non-crystallizing material (potassium salts, proteins, gums, other carbohydrates, lipids and aromas) into quite concentrated domains with poor entrapment capacity. Growing crystals can possibly also open closed biological structures and thereby contributing to aroma loss. It is likely that these processes explain the events after 240 minutes of drying. Our intention with the microstructural investigation was to prove this explanation. However, it was not possible to get sufficiently clear evidences by these observations. Thus we selected a different strategy to analyze the possibilities for a microstructural change.

We assume that the system is a suspension of insoluble fibers in an aqueous saccharide solution. This simplistic model of the fruit pulp can be compared with compositional data from Magaia [22]. The remaining water during the drying process is distributed between the fiber component and the aqueous solution phase. Assuming equilibrium between these both phases we estimate the availability of water for keeping the saccharides dissolved from the water activity. The solubility is of the saccharides mixture of the *Vangueria infausta* (50% sucrose, 25% glucose and 25% fructose [22] is obtained by comparing with the phase diagrams of sucrose, fructose and glucose [23-25], redrawn in Figure 5. By interpolating in the almost fully linear phase diagram and by using the solubilities of sucrose, fructose and glucose [24, 25] we could observe that the sucrose and glucose hydrate are the saccharide that are expected to crystallize first during the drying process. However, when the temperature is risen, the glucose hydrate dissolves and the solubility of glucose increases more rapidly than the solubility of sucrose leading to an expansion of area *a* in Figure 5. Thus, it can be expected that pure sucrose will be the first saccharide solid that will precipitate during the drying process.

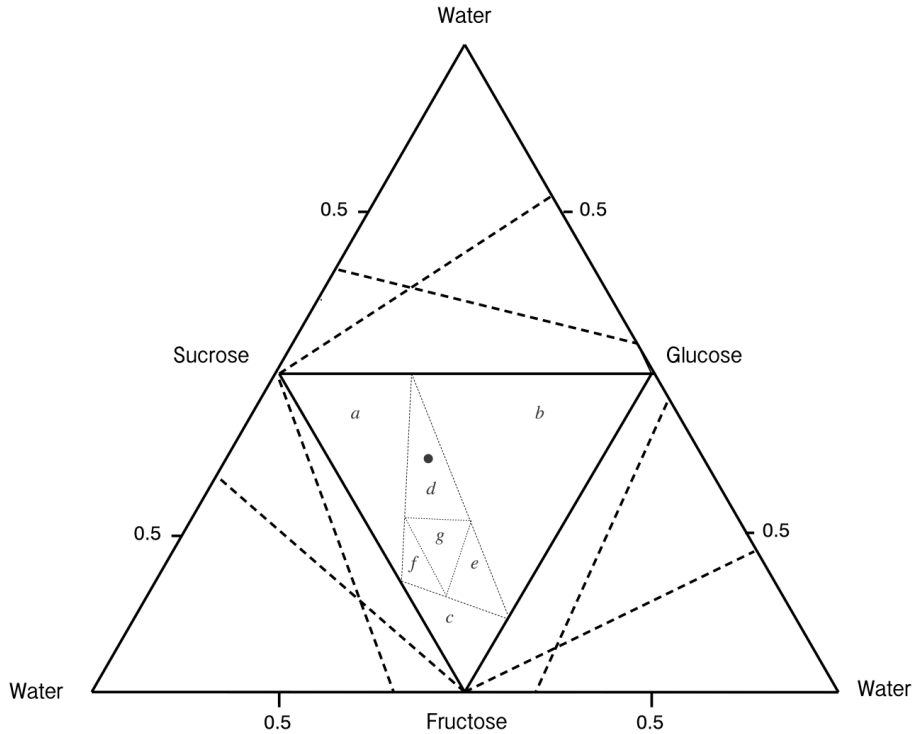

Figure 5. Simplified phase diagram of sucrose-glucose-water, sucrose-fructose-water and fructose-glucose-water at 30°C. The inner triangle represents the composition of the precipitated solid phase from a quaternary water-sucrose-glucose-fructose solution of a given sugar composition. The first precipitate is in area *a*, sucrose, *b*, glucose hydrate, *c*, fructose, *d*, sucrose and glucose hydrate, *e*, glucose hydrate and fructose, *f* sucrose and fructose, *g*, sucrose, glucose hydrate and fructose. The dot marks the composition of the saccharides in the *Vangueria infausta*. Redrawn after data from [24, 25].

The water availability to the saccharide solution phase is shown by the water activity. The water activity at the solubility limit of sucrose in the sucrose-fructose-glucose solution can be compared with the water activities during the drying and be used to estimate the likeliness for crystallization of the sucrose during the drying process.

The activity of water in the binary solutions of saccharides is used to obtain an estimation of the solubility and activities of the saccharides. An extrapolation from binary systems into a quaternary water-sucrose-glucose-fructose system is used to estimate the solubility of sucrose and the water activity using numerically integrating of the Gibbs-Duhem equations as described in the Appendix. The result of this estimation is a curve providing water activity at the sucrose crystallization limit as a function of temperature. In Figure 6 the water activity and temperature at the crystallization limit as obtained from the procedure in the Appendix is compared with the drying trajectory (water activity and sample temperature during the drying

process). As a reference the water activity [27] and temperature at the phase boundary [25] of the binary sucrose-water system is included.

Figure 6 shows that the water activity initially is high (0.97) and that the temperature is low. After 60 minutes of drying the a_w starts to decrease and the sample temperature to increase. No crystallization can be expected yet. Gradually, during the progress of the drying process, the water activity decreases. When the drying trajectory passes the solubility curve we may expect the system to crystallize.

The results indicates that it is likely that sucrose crystallizes after about 120-180 minutes of drying and it is expected that crystallization may lead to loss of aroma during drying process [6]. However, one may always speculate about if the crystallization occurs or if the system remain super-cooled. It is possible that the presence of crystalline cellulose may act as nucleation sites and reduce super-cooling. It is also possible that there are concentration gradients that may initiate crystallization. On the other hand, it is also possible that soluble fibers my repress the crystallization as this fraction is high [13,26]. The further evaluation of these effects is difficult, as the specific chemical nature (pectin, galactanes) as well as the solubilization at a molecular level (most likely low) of the fiber is not known.

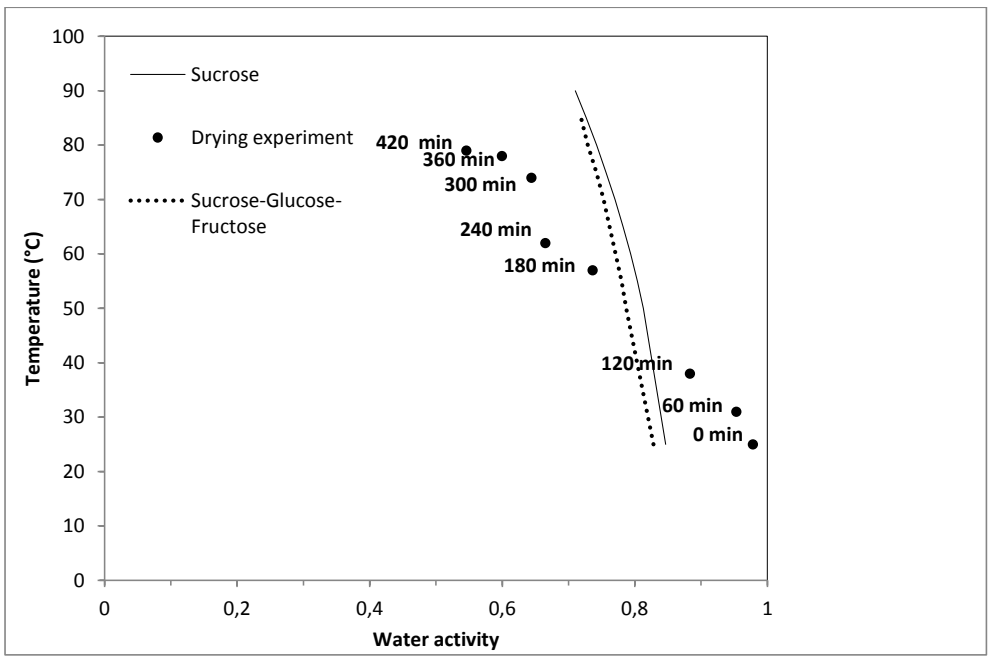

Figure 6. Relationship between the water activity and sample temperature during the drying process of fruit pulp from *Vangueria infausta* (filled dots). The result is compared with the water activity at the phase boundary for crystallization of sucrose in water (using the a_w model by Starzak and Mahtlouthi [27] and solubility data from [25]) and the water activity at the phase boundary for crystallization of sucrose from a solution of 50% sucrose, 25% fructose and 25% glucose as estimated in the appendix.

6. Conclusions

This work shows the effect of convective drying on aroma retention of fruit pulp from *Vangueria infausta* when subject to isothermal drying at 80 °C. A loss of aromas has been observed at a water activity below 0.65. It seems likely the crystallization of sucrose and the increasing object temperature is the most critical factors for of the loss volatile retention.

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Appendix

Estimation of the water activity at the solubility limit of sucrose in the tertiary mixture of sucrose, fructose and glucose using Gibbs-Duhems equation.

Gibbs-Duhems equation was used to estimate the water activity at the solubility limit of the sucrose in the quaternary mixture of water, sucrose, fructose and glucose. The starting point was the solubility limit in the binary systems [25]. At the solubility limit the aqueous solution is in equilibrium with one pure solid phase, which is considered the reference condition of the sugars. The activities of the sugars in the binary solutions as a function of mole fraction water were obtained by a numerical integration of equation (2) using a recursion approximation (3) as suggested by Hempel [26].

$$x_s \cdot d\ln a_s = -x_w \cdot d\ln a_w \tag{1}$$

$$\ln a_s(x_w) = -\int_{\ln a_w(x_w^*)}^{\ln a_w(x_w)} \frac{x_w}{x_s} \cdot d\ln a_w \tag{2}$$

$$\ln a_{s,i+1} = \ln a_{s,i} + \frac{(\ln a_{w,i+1} - \ln a_{w,i}) \cdot (x_{w,i} + x_{w,i+1})}{x_{s,i} + x_{s,i+1}} \tag{3}$$

The water activities for sucrose at different temperatures were estimated using the equation suggested by (Starzak and Mahtlouthi [27] while water activities as a function of concentration at 25°C for glucose and fructose was obtained using the approximation by Miyawaki [28].

By using the numerical integration (3) the activity and the activity coefficients of sucrose was obtained as a function of concentration and temperature. For fructose and glucose the activity and activity coefficients were only obtained at 25°C and we had to assume them to be less temperature dependent.

$$\sum_j^n x_{s_j} \cdot d\ln a_{s_j} = -x_w \cdot d\ln a_w \tag{4}$$

$$\ln a_w(x_w) = -\int_{\sum_j^n d\ln a_{s_j}(0)}^{\sum_j^n d\ln a_{s_j}(x_{s_j})} \frac{\sum x_{s_j}}{x_w} \cdot \sum_j^n d\ln a_{s_j}(x_{s_j}) \tag{5}$$

$$\ln a_{w,i+1} = \ln a_{w,i} + \frac{(\sum \ln a_{s_j,i+1} - \sum \ln a_{s_j,i}) \cdot (\sum x_{s_j,i} + \sum x_{s_j,i+1})}{x_{w,i} + x_{w,i+1}} \tag{6}$$

$$a_{s_j} = \gamma_{s_j} \cdot x_{s_j} \; where \; \gamma_{s_j} \sim \gamma_s(x_w) \tag{7}$$

The activity coefficients of the sugars in the mixture are assumed to be approximately equal to the activity coefficient in the corresponding binary mixture with the same mole fraction water, as suggested by Walstra [29]. Thus, the activity coefficient of the saccharides in the complex tertiary mixture (0.5 sucrose, 0.25 glucose and 0.25 fructose (mass fraction)) was obtained by using the approximation that the activity coefficient is determined by the mole fraction water

and thereby can be obtained from the binary data obtained when equation (2) was solved as described in equation (7). The water activities are obtained by integrating (5) using the recursion procedure in (6) until the activity of 1 is obtained. The results are summarized in Table 2.

The approximations used in this estimation are obviously rather rough. But, by comparing with scattered experimental observations we think the results are not too far from the what would be obtained with stricter assumptions.

Table 2. The results of the numerical procedures aimed to obtain an approximate value of the sucrose and total solute concentrations and water activities when crystallization may be induced in a sucrose-glucose-fructose system. The composition of the dry matter is a mass fraction of 0.5 for sucrose, 0.25 for glucose and fructose respectively. *C* is solubility in mass fraction, *X* solubility in mole fraction and a_w the predicted water activity.

T	$C_{sucrose}$	$C_{solutes}$	C_{water}	$X_{sucrose}$	X_{solute}	X_{water}	a_w
25	0.319	0.637	0.363	0.040	0.119	0.881	0.828
50	0.344	0.687	0.313	0.048	0.145	0.856	0.787
55	0.349	0.697	0.303	0.050	0.151	0.850	0.780
60	0.355	0.710	0.290	0.053	0.159	0.842	0.771
65	0.362	0.724	0.276	0.056	0.168	0.833	0.762
70	0.367	0.735	0.265	0.059	0.176	0.825	0.753
75	0.374	0.748	0.252	0.062	0.186	0.815	0.742
80	0.380	0.761	0.239	0.066	0.197	0.804	0.729
85	0.385	0.771	0.229	0.069	0.206	0.795	0.719

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