Saffron (*Crocus sativus*): Post Harvest Technology for Optimising Spice Quality – and New Product Potential

by

Matthew Gregory

(BSc University of Tasmania)

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Matthew Gregory

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ABSTRACT

ISO and alternative analytical techniques were applied to Australian saffron. These analysis methods were used to assess quality in comparison to the international standard and products from other major producing countries. While Australian saffron was generally shown to be of high colour quality, also identified were the potential for greatly improving aroma strength and the risk of insufficient drying leading to degradation of components during storage.

The deficiencies of the ISO method for determination of aroma strength have been referred to previously by a number of authors. In this study statistical proof of the inaccuracy of the aqueous extraction and absorbance measurement was demonstrated for the first time. Dual polarity extractions and chromatographic techniques were therefore used for all measurement of saffron components in subsequent experiments.

Experiments were conducted to investigate the optimisation of post harvest treatment of stigmas for maximizing aroma generation and colour retention. Critical to this are conditions of stigma drying during which aroma is generated. In Australia, saffron is dried at moderate temperatures (40-50 °C) with airflow over approximately 90-120 minutes. Elsewhere a great variety of methods and conditions are used without any consensus on what constitutes optimal drying conditions. Moreover, doubts about the previously accepted chemical mechanism for safranal (the major aroma compound) generation together with proposed alternative pathways have recently surfaced.

These studies showed that drying saffron at elevated temperature (≈ 90 °C) produces significantly more safranal while also enhancing colour strength. This latter effect was shown to be due both to greater retention of crocin pigments (from prevention of enzymatic degradation of these pigments) and increased availability of crocins (due to changes in inter- and intra-cellular structure). The use of airflow to speed drying was shown to be detrimental to aroma content.

For the first time the heating profile of stigmas was measured to show that evaporative cooling is a significant factor during drying. Humidification of the drying chamber was shown to slow the rate of filament drying while also reducing evaporative temperature suppression. This prolongs the period that stigmas remain within an intermediate range of water activity where hydrolysis of the glycoside (picrocrocin) to form the volatile aglycon (safranal) is favoured at relatively high temperature (90 °C). It also causes the stigmas to heat more rapidly to a temperature at which this conversion occurs and also where enzymatic pigment degradation is de-activated.

Under these conditions at least 3 fold increases in safranal production were demonstrated without the need for alternative generation mechanisms via thermal crocin degradation to explain safranal gains. Drying at moderate temperatures (< 60 °C) involves the action of β-glucosidase on picrocrocin to produce an intermediate compound, followed by subsequent dehydration to form safranal. It was shown that where enzyme activity had produced a significant amount of the intermediate, the end product was not safranal when the subsequent drying was at higher temperatures. This provides a possible explanation for observations of picrocrocin loss without corresponding safranal gain that has led others to doubt the previously accepted safranal production pathway.

It was proposed that while the formation of safranal from crocins may also occur at very high temperatures (>> 100°C), this process is relevant only to the culinary use of the spice rather than as a factor during drying. Notwithstanding this, the conditions of drying could well determine the potential for subsequent aroma formation during cooking.

Studies of the cellular structure and porosity of saffron demonstrated how different drying conditions affect both the availability of pigments for aqueous extraction and filament strength. Drying at high temperature was shown to enhance the dissolution of crocins, but also increase brittleness. Elevated humidity drying prevented this brittleness by reducing intercellular spacing (porosity) while maintaining the enhanced colour availability. It is proposed that thermal cell membrane disruption (allowing rapid crocin dissolution) had occurred independent of humidity determined cell shrinkage rates.

Distillations and extractions were undertaken to assess the potential for new products from the waste flower parts (petals and stamens). The best fragrance product obtained was a non-polar extract, though it required a small content of stigmas included with the flowers extracted to give it aroma "impact". This product had a vivid yellow/orange colour derived from its content of carotenoid fatty acid esters. These carotenoids were identified as di-esters of lutein isomers.

Mid-polarity extracts of waste flowers were shown to contain a high content of flavonoids, particularly glycosidic conjugates of kaempherol; compounds which are known to be potent antioxidants.

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Abbreviations

Abs	Absorbance
AU	Absorbance units
A_w	Water activity
FID	Flame ionisation detector
GC	Gas chromatography
HCC	hydroxy-β-cyclocitral (4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde
HPLC	High performance liquid chromatography
HPLC- UV-Vis	High performance liquid chromatography with absorbance detection in ultra violet – visible wavelength range.
LC-MS	Liquid chromatography – mass spectroscopy
LCQ	Liquid chromatograph quadrapole (MS)
MS	Mass Spectroscopy
MW	Molecular weight
RH	Relative humidity
RT	Retention time
SEM	Scanning electron microscope(y)
SIM	Selected ion monitoring mode
UV-Vis	Ultraviolet – visible light (absorbance detector)
W/w	Weight per weight
wt.	Weight

1 LITERATURE REVIEW

1.A Background

1.A.1 What is saffron ? – biology

Saffron spice consists of the dried stigmas from the flowers of *Crocus sativus*. This Iridaceous species consists of a corm (bulbous underground starch storage organ) up to 45 mm in diameter from which 5-7 narrow leaves (up to 40 cm in length) emerge after the aestivation period in autumn; the plant being dormant over summer. Flower buds appear either concurrently or soon after appearance of the first leaves and extend approximately 3-7 cm above ground.

The flowers have 6 mauve to lilac coloured tepals (combined petals and sepals), 3 yellow stamens and bright red, tripartite stigmas consisting of three tubular filaments joined at the pistil (see figure 1.1). The mature stigmas may reach approximately 15 – 40 mm in length as the flowers open, which is usually within 24 hours of the flower bud emerging above ground[*1-3*].

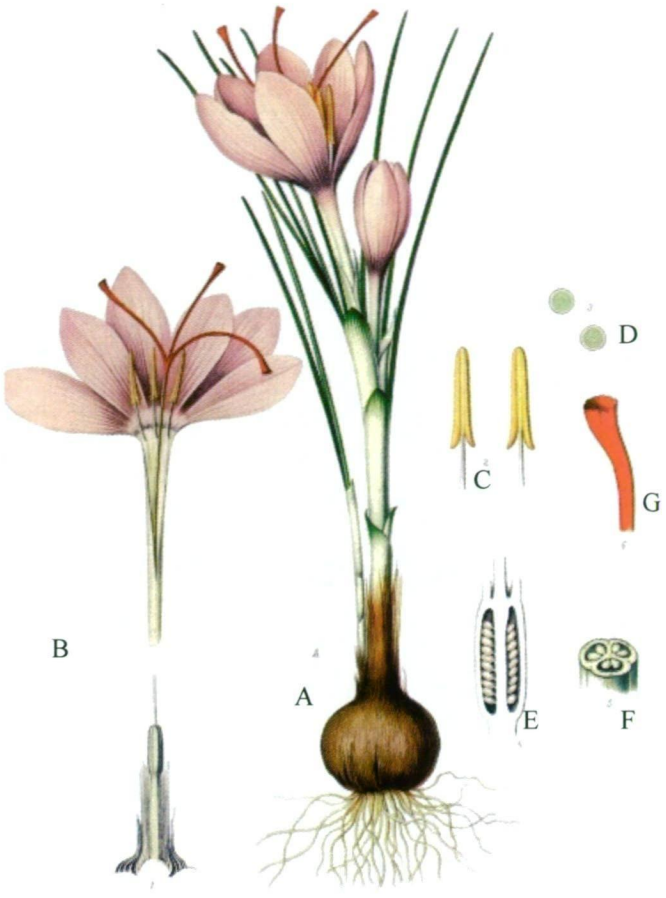
The plant is a triploid and sterile as the flowers fail to produce viable seeds and thus propagation is by vegetative growth[*1, 3, 4*]. Multiplication of the corms is to some extent dependent on human assistance as the small "daughter cormlets" must be dug up and separated every few years to allow full growth potential to be realised[*2, 5*].

1.A.2 Origins of the spice

Domesticated saffron crocus (*Crocus sativus*) is thought to have been derived from *Crocus cartwrightianus* which occurs in the wild in the eastern Mediterranean and Asia Minor[*1*]; a proposition supported by DNA analysis[*6*]. It is thought that the spice was first harvested from this wild species and evidence of the use of saffron pigments in cave art has been dated at 50,000 years, while medicinal and culinary uses date to the ancient Sumerians before 1,700 BC in what is now Iraq [*7, 8*].

The first images of saffron collection in Aegean Bronze Age art date the appearance of the spice in Greek/Minoan culture possibly as early as 3,000 BC[*7*], but more definitely in Crete by the 17th century BC[*1, 4*]. The dating of the domestication of saffron is a matter of some conjecture, but the best evidence suggests that this had occurred by the 4th century BC[*1, 6*]. What is accepted is that the selection and subsequent cultivation of sterile triploid mutants (distinguished by elongated stigmas) from the wild species, resulted in the modern saffron plant (*Crocus sativus*)[*1, 4, 7, 8*].

From the Mediterranean region, saffron cultivation spread to northern India (particularly Kashmir) and then China; and although there are wildly conflicting accounts of when this occurred, it was certainly by the 13th Century AD [*6, 7*]. Cultivated saffron was introduced to Spain by the Moors in or before the 10th century AD and then spread to the rest of Europe during the middle ages, particularly in association with the Crusades [*6, 7, 9*].



A – Whole plant including corm

B – Profile of flower

C – Stamens

D – Pollen

E – Lateral profile of carpel showing ovaries

F – Transverse profile of ovaries showing ovules

G – Stigma (and upper part of style)

Figure 1-1: Crocus sativus morphology (From Koehler's Medicinal-Plants 1887)

1.A.3 Traditional uses

Possessive of a distinctive yet "subtle, sensual and seductive" aromatic odour combined with a pleasant bitter taste and a unique colouring strength, saffron is a widely used traditional ingredient in Iranian, Arab, Central Asian, Indian, Mediterranean, Central European and even Cornish cuisines[*6-8, 10*]. It is used in both savoury and sweet dishes including liquors, with the relative importance of the aroma and colouring properties varying with recipe[*8*].

Saffron is regarded as the most powerful naturally occurring water soluble dye with the "potential to colour up to 10,000 times its own weight a strong shade of yellow"[*8*]. The most widespread and common use of saffron as a dye has been (and is) in India and China (and related

cultures) where it has religious significance and is even used in cosmetics, although its use in textiles and artworks extends to many other cultures[*6, 7, 10, 11*] and it has been used in histological stains[*1*].

From ancient times saffron has been used as a health promoter and drug for treatment of a wide spectrum of medical complaints and the biological properties of the spice have appeared in the pharmacopoeias of many countries over centuries[*1, 4, 7, 8*]. It has been traditionally used in various cultures but particularly in China to treat conditions including colic, insomnia, depression, anxiety, asthma and coughs, thrombosis, flatulence and as a blood anticoagulant and thinning agent[*1, 12, 13*].

Saffron has also been used in treatments for various skin disorders including as a nutritional therapy for psoriasis[*6, 8*] as an anti-inflammatory[*14*], an aphrodisiac[*10*] and to treat heart disease[*1*]. However, some of the most significant historical therapeutic uses relate to the anti-carcinogenic, anti-mutagenic and anti-oxidant properties of various preparations of the spice[*1, 6, 7, 12, 15*].

The toxicity of saffron at doses of 0.5g or more is well established although the mechanism of this not well understood[*10, 15, 16*].

1.A.4 International production and markets

Saffron is currently known to be commercially produced in a variety of countries including: Iran, India, Spain, Italy, France, Switzerland, Greece, Pakistan, Afghanistan, Morocco, United Arab Emirates, Egypt, Turkey, Israel, Azerbaijan, China, Australia and New Zealand[*1, 6, 17*]. For centuries Spain was regarded as the leading producer and exporter of saffron in the world (both in terms of quality and volume), but in recent decades production there has rapidly declined from approximately 29 tonnes[*1*] to less than 1 tonne per annum; the remainder now subject to culturally related trade protection[*6*].

On average it requires 150-250,000 plants to produce 1 kg of saffron; the value of which may vary widely from approximately $1,000 up to as much as $100,000 USD depending on the source and market[*1, 5, 6, 10, 17*]. The annual world production of saffron was officially estimated to be 100-180 tonnes at an average value of $7000 USD/kg prior to 2004[*18, 19*], but more recent estimates have put the total at around 205-275 tonnes, of which Iran is reported to now produce 80%; a figure projected to rise in coming years[*6, 17*]. The primary reason for this growing dominance is the relative costs of production for a crop that is labour intensive[*1, 2, 6, 10*].

The main importers of saffron include: Italy, Spain, Switzerland, France, Germany, The United Kingdom, Sweden, Saudi Arabia, India, Pakistan, The United States and China[*17*].

1.A.5 Australian production and market

Australia imports approximately 800 kg of saffron annually at a value of A$14m[*19*], while the retail value(on the local market) of 1 kg of Australian produced saffron is approximately A$100,000 [*2, 5, 19*].

Tas-Saff Pty. Ltd. is the first and only significant commercial producer of saffron in Australia. With some 34 growers in Victoria, NSW and Tasmania (as of 2006), it has been marketing product locally for 15 years[*19*].

1.B Saffron chemistry

1.B.1 Carotenoid precursors

The major colour, flavour and fragrance components of saffron are thought to be secondary metabolites from the biodegradation of the C_{40} carotenoid, zeaxanthin (see figure 1-2). Besides zeaxanthin, other carotenoids reported in saffron include C_{40} compounds β-carotene, γ-carotene, σ-carotene, phytoene, phytofluene lycopene, lycopersene and tetrahydro-lycopene[*4, 9, 20*].

1.B.2 Formation of the main flavour, aroma and pigment components

The glycosylated forms of the C_{20} carotenoid crocetin (see figures 1-2 &1-3), which are collectively referred to as the crocins, (of which crocin, the digentiobiose ester of crocetin, is the most abundant), are the principal yellow pigments in saffron, while picrocrocin is regarded as the main taste factor (see figure 1-2)[*9, 21*].

Because of the presence of C_{40} carotenoids and the absence of other C_{20} carotenoids (as possible crocetin precursors), it has been concluded that the formation of the crocetin moiety must occur via degradation of a C_{40} carotenoid rather than by an addition process (eg dimerisation of C10 compounds). This carotenoid was assumed to be zeaxanthin as it is the only C_{40} carotenoid present with the appropriate stereochemical configuration to produce picrocrocin[*4, 9, 22*].

It was been proposed that the oxidative degradation of zeaxanthin to from the major flavour, aroma and colour components occurs via a hypothetical 7,8-carotenase enzyme cleaving the zeaxanthin molecule at either end of the polyene chain to produce 1 molecule of crocetin and 2 molecules of picrocrocin (see figure 1-2)[*9, 20, 23, 24*]. Recent studies have confirmed this and provided much insight into the enzymatic pathways and genetic control of both the accumulation and degradation of zeaxanthin to produce crocetin and its subsequent glycosylation to form the various crocetin esters (crocins) during stigma development[*21-23, 25-27*]. The structures of the main (most abundant) crocins are illustrated in figure 1-3.

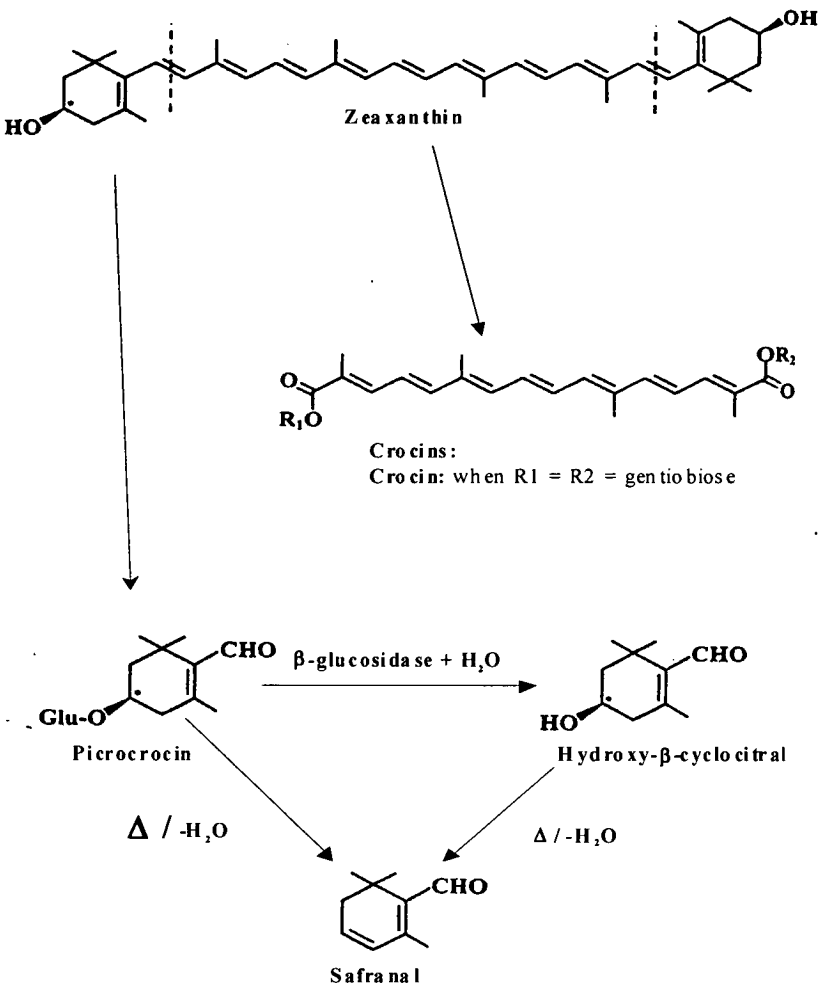
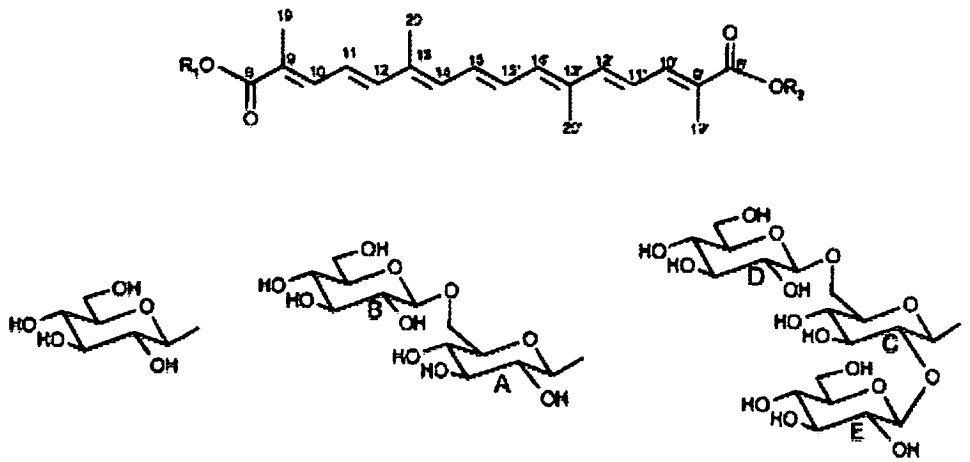


Figure 1-2: The accepted pathway for zeaxanthin biodegradation to produce principal secondary metabolites responsible for colour (crocins), flavour (picrocrocin) and aroma (safranal) in saffron (scheme drawn with reference to an analysis of reviews in the literature[*4, 9, 28-30*]).

The odourless, colourless and water soluble picrocrocin, itself a glycoside, has been generally regarded as the main taste component responsible for the bitter flavour of saffron[*4, 9*]. While the compound is certainly bitter and would contribute significantly to the taste, Carmona and Alonso[*28*] rightly highlight the lack of scientific evidence directly correlating picrocrocin concentrations with the degree of saffron bitterness and point out that significant levels of other compounds such as flavonoids may also contribute to this taste.

It is also generally accepted that picrocrocin is the precursor of safranal, the main odour component of saffron (usually >60% of volatile content)[*4, 9, 10, 31*], which is only produced in the stigmas upon drying. This conversion (see figure 1-2) is thought to occur by alternative pathways: Firstly via an intermediate, 4R-hydroxy-β-cyclocitral (HCC (4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde), which is produced by the enzymatic action of β-glucosidase at moderate temperatures and then readily dehydrated to form safranal. Secondly it may occur by direct (non-enzymatic) hydrolysis/dehydration of picrocrocin to form safranal under more extreme conditions such as high temperatures or extreme pH[*9, 32, 33*].



X Y Z

1: $R_1 = R_2 = H$, Crocetin

2: $R_1 = H$, $R_2 = X$, Crocetin-mono-(β-D-glucosyl)-ester

3: $R_1 = R_2 = X$, Crocetin-di-(β-D-glucosyl)-ester

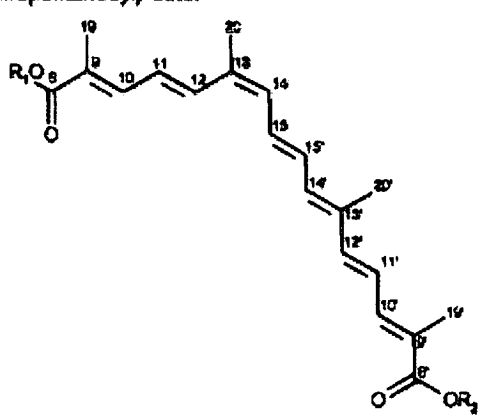
4: $R_1 = H$, $R_2 = Y$, Crocetin-mono-(β-gentiobiosyl)-ester

5: $R_1 = X$, $R_2 = Y$, Crocetin-(β-D-glucosyl)-(β-gentiobiosyl)-ester

6: $R_1 = R_2 = Y$, Crocin [Crocetin-di-(β-gentiobiosyl)-ester]

7: $R_1 = Y$, $R_2 = Z$, Crocetin-(β-gentiobiosyl)-(β-neapolitanosyl)-ester

8: $R_1 = R_2 = Z$, Crocetin-di-(β-neapolitanosyl)-ester



9: $R_1 = R_2 = Y$, 13Z-crocin

Figure 1-3: Crocetin esters (crocins) configurations reported in saffron as illustrated by Pfister et al (1996)[39]. The type of glucoside group (R) may be X, Y or Z. All of these structures may have *cis* or *trans* configuration (i.e. structure 9 is an example of *cis*), giving a total of 16 distinct esters. Further variations are possible however as the 3 glucose groups may be either neapolitanose or triglucose in structure [6;38-40]. Considerable variation and even confusion exists in regard to the common names for the different esters. The following nomenclature is used here for the crocin esters generally reported as the most abundant in saffron – (in order of abundance) trans and cis isomers of 4-crocin, 3-crocin and 2-crocin, which correspond above to the structures 6, 5 and 4 respectively[10;39;41].

The production of safranal from picrocrocin by either pathway, including the conditions favouring such generation, is not fully understood. Studies [*28, 34*] where previously dried stigmas were then subjected to heat treatments that resulted in almost complete loss of picrocrocin; but did not result in corresponding safranal production, indicate that the proposed model may be more

complicated with other factors involved to the conversion process. Either the heat treatments also resulted in complete degradation of any safranal formed or alternative pathways are possible with products (other than safranal) formed from the degradation of picrocrocin. There have been suggestions that the generation of safranal may be linked to water activity[*35, 36*]. Furthermore, it has been demonstrated by Carmona *et al* [*29, 37*] that safranal may be generated at high temperatures (>100 °C) without a corresponding reduction in picrocrocin, and that this may occur even when picrocrocin has previously disappeared[*38*].

Carmona et al [*29, 37*] have postulated a mechanism where, besides the accepted conversion pathway from picrocrocin, safranal may also be generated by thermal oxidative degradation of crocins (see figure 1-4) and they propose that this may represent an accelerated version of what happens during the normal aging process in the spice. While the detection of compounds that could be intermediate in the transition of the crocetin moiety to form safranal provides evidence that this may occur, the temperatures involved (treatment at 130 °C followed by analytical sampling at up to 240 °C) in the studies were significantly higher than what would normally be used to dry saffron. It is uncertain whether this would occur during aging (storage) at normal temperatures (<<100 °C). Rather it may only have relevance to changes that occur during certain cooking conditions. Even if it does occur very slowly during aging, such degradation and thus loss of crocins (i.e. pigments) would not be desirable for optimum saffron quality.

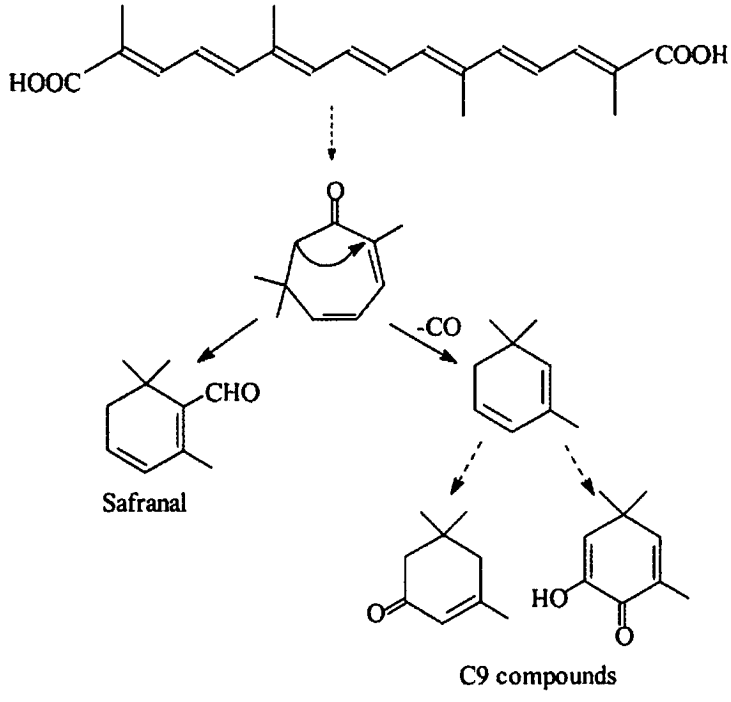


Figure 1-4: Mechanism for thermally induced conversion of crocetin moiety (and thus crocins) to produce safranal (and other possible aroma compounds observed in saffron) as proposed by Carmona *et al*[35].

1.B.3 Other aroma compounds and overall olfactory contribution

Besides these major components there are many other potential aroma factors in saffron with over 100 volatile compounds having been identified[*9, 29, 31*]. These include 25 carotenoid derived compounds that have been shown to contribute specific notes to the overall odour of the product (see figure 1-5). These may be produced as degradation products of safranal, HCC and/or picrocrocin, or from alternative carotenoid degradation pathways[*31, 39-41*] – either directly from zeaxanthin, or (possibly) from the thermal degradation pathway of crocins referred to above[*37*](see figure 1-4). It is very likely that some of these compounds are only produced upon cooking the spice, as some of the extraction and analysis techniques used in these identifications involved elevated temperatures (eg via distillation or thermal desorption for Gas Chromatography)[*4, 29, 42*]. Whether these thermally derived products should be regarded as analysis artefacts or representative of saffron when used in cooking is open to interpretation.

There are somewhat conflicting reports as to which compounds (other than safranal) contribute most to the overall aroma of saffron. Moreover, the composition of the such compounds can vary greatly between samples of different origin[*29, 39, 41, 43*]. Tarantilis and Polissiou[*31*] in their study which compared different extraction techniques (with and without heat), determined that of some 23 compounds identified, 5 (including safranal and isophorone) where characteristic of saffron aroma (compounds 2, 4, 6, 7 and 9 in figure 1-5 and table 1-1), but did not describe their olfactory contribution.

Separate GC-olfactometry studies of saffron samples by Rödel and Petrizka[*44*] and Cadwallader *et al*[*41*] both highlighted that a relatively small number of those compounds originally detected and identified by others, were actually significant contributors to saffron aroma; but there was little agreement between these authors as to which notes were attributable to which compounds. The latter study also involved aroma extract dilution analysis, where they identified safranal and 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one (an isomer of isoporone) as being the most important, with the latter claimed to have a slightly higher aroma contribution. Both were attributed with characteristic saffron notes. Knapp *et al* [*39*], however, while confirming that this compound was important, found that it was of secondary importance to safranal (and equivalent to isophorone in olfactory impact). Among other authorities, the consensus is that safranal is the most important aroma compound in saffron, both qualitatively and due to its high relative abundance[*10, 29, 31, 33, 42, 45, 46*].

Other compounds reported to be important in saffron aroma include HCC (spicy note), 2-phenylethanol (flower note), linalool (flower note), 3-methylbutanoic acid (sour-rotten) and acetic acid (sour-vinegar)[*9, 29, 42*]. There are also many additional compounds formed through lipid oxidation or hydrolysis of non-carotenoid precursors that have been identified as minor constituents of saffron, but which may also contribute to the overall fragrance[*9, 10*]. Glycoside precursors of both carotenoid and non carotenoid derived volatiles appear to be present in the Crocus stigmas before, and in some cases, after drying[*39, 47*], suggesting that there is a complex array of factors which can subtly influence saffron quality through liberation of volatiles during drying, storage or cooking.

While differences in the amounts of these and other compounds may be useful in discriminating saffron from different sources including different drying and storage techniques[*42, 48, 49*]; and expert assessments designating relative desirability of specific aroma notes are available (sweet,

spicy and floral being good and woody, sour and herbal notes being bad)[*45, 50-52*]; there is no consensus amongst authors as to what constitutes a desired aroma compound profile. The only accepted view is that safranal is the most important aroma compound and that its content should be as high as possible to optimise spice quality[*10, 29, 31, 33, 42, 46*].

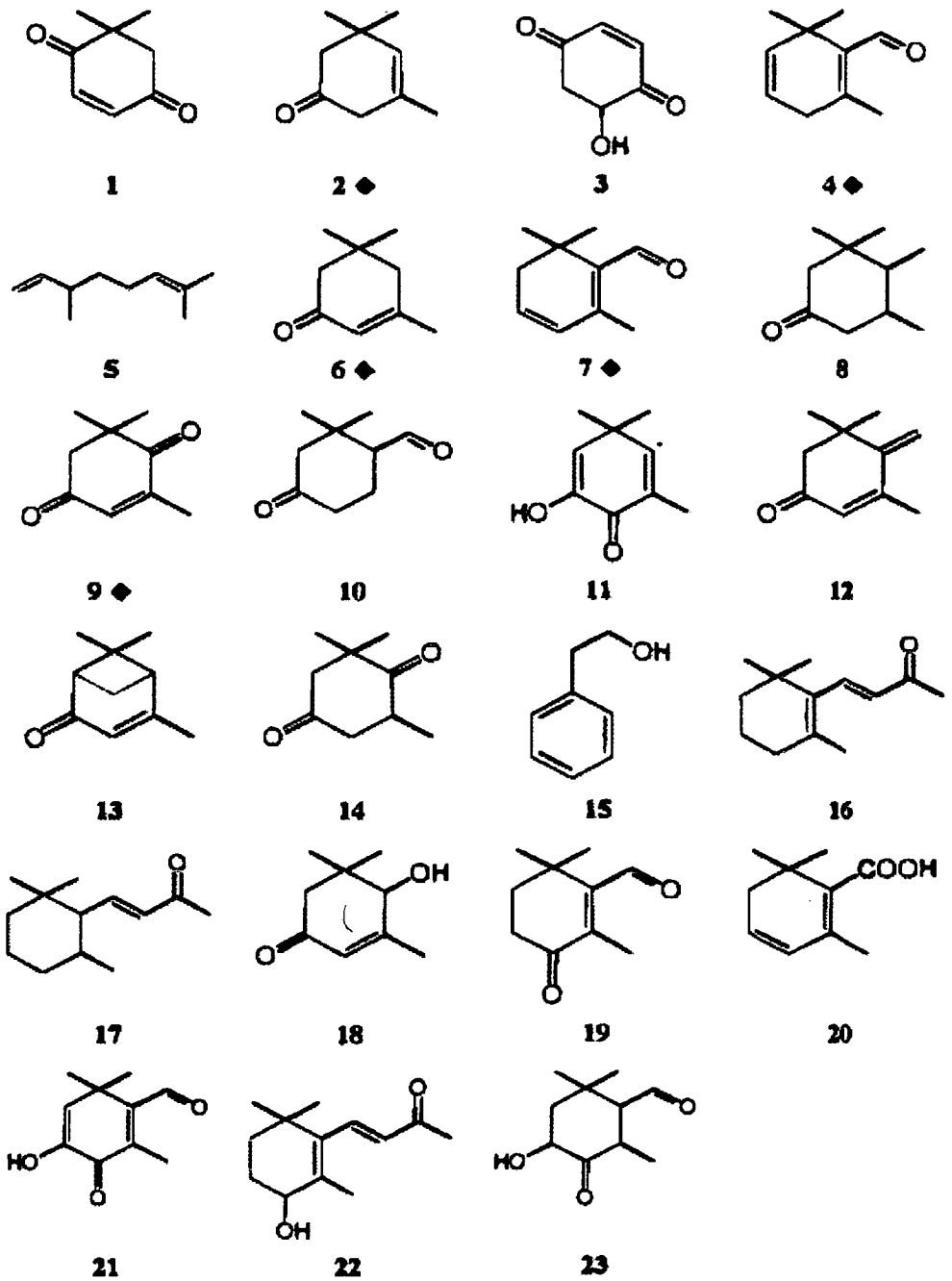


Figure 1-5: Molecular structures of some aroma compounds detected in saffron – as illustrated by Tarantillis and Polissiou 1997[*31*](compound numbers correspond with names given in table 1-1; ♦ = characteristic of saffron aroma.)

Table 1-1: Chemical identification and relative abundance of saffron aroma compounds detected by 3 different sampling methods: MSDS = Micro-simultaneous distillation extraction, VHS vacuum headspace sampling and SD steam distillation. Quantification units are relative area of total ion chromatogram. (from Tarantilis and Polissiou 1997[*31*])

			Isolation method (relative area of TIC)		
Structure no.	Compound	molecular formula	MSDE	VHS	SD
1	5,5-dimethyl-2-cyclohexen-1,4-dione	$C_8H_{10}O_2$ MW:[a] 138	-	-	4.7
2	3,5,5-trimethyl-3-cyclo-hexen-1-one	$C_9H_{14}O$ MW: 138	28.7	-	5.4
3	2-hydroxy-5-cyclohexen- 1,4-dione	$C_6H_6O_3$ MW: 126	-	-	5.9
4	2,6,6-trimethyl-1,4- cyclo-hexadien-1-carb- oxaldehyde	$C_{10}H_{14}O$ MW: 150	34.4	13.4	6.9
5	3,7-dimethyl-1,6-octadiene	$C_{10}H_{18}$ MW: 138	38.4	-	
6	3,5,5-trimethyl-2-cyclo-hexen-1-one (isophorone)	$C_9H_{14}O$ MW: 138	40.8	16.2	7.3
7	2,6,6-trimethyl-1,3-cyclo-hexadien-1-carbox- aldehyde (safranal)	$C_{10}H_{14}O$ MW: 150	44.9	17.3	9.3
8	3,3,4,5-tetramethylcyclo-hexan-1-one	$C_{10}H_{18}O$ MW: 154	45.2	-	
9	2,6,6-trimethyl-2-cyclo-hexen-1,4-dione	$C_9H_{12}O_2$ MW: 152	47.1	14.2	7.8
10	2,2-dimethyl-4-oxocyclo-hexan-1-carbox- aldehyde	$C_9H_{14}O_2$ MW: 154	-	-	7.9
11	2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien- 1-one	$C_9H_{12}O_2$ MW: 152	-	-	8.2
12	4-methylene-3,5,5-tri-methyl-2-cyclohexen- 1-one	$C_{10}H_{14}O$ MW: 150	49.1	18.0	9.7
13	4,6,6-trimethyldicyclo-[3.1.1]hept-3-en-2-one	$C_{10}H_{14}O$ MW: 150	49.3	18.1	-
14	2,6,6-trimethylcyclohexan-1,4-dione	$C_9H_{14}O_2$ MW: 154	52.1	14.9	-
15	2-phenylethanol	$C_8H_{10}O$ MW: 122	59.7	13.2	-
16	4-(2,6,6,-trimethyl-1-cyclo-	$C_{13}H_{20}O$	61.5	-	-

			Isolation method (relative area of TIC)		
Structure no.	Compound	molecular formula	MSDE	VHS	SD
	hexen-1-yl)-3-buten- 2-one	MW: 192			
17	4-(2,2,6,-trimethyl-cyclo-hexan-1-yl)-3-buten-2-one	$C_{13}H_{22}O$ MW: 194	62.4	-	-
18	4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one	$C_9H_{14}O_2$ MW: 154	-	-	10.4
19	2,6,6-trimethyl-3-oxo-1-cyclohexen-1-carboxaldehyde	$C_{10}H_{14}O_2$ MW: 166	-	-	11.9
20	2,6,6-trimethyl-1,3-cyclo-hexadien-1-carboxylic acid	$C_{10}H_{14}O_2$ MW: 166	-	-	12.5
21	4-hydroxy-2,6,6-trimethyl-3-oxo-1,4-cyclohexa-dien-1-carboxaldehyde	$C_{10}H_{12}O_3$ MW: 180	-	-	13.9
22	2,4,4-trimethyl-3-(3-oxo-1-butenyl)-2-cyclohexen- 1-ol	$C_{13}H_{20}O_2$ MW: 208	-	-	14.2
23	4-hydroxy-2,6,6-trimethyl- 3-oxo-cyclohexan- 1-carboxaldehyde	$C_{10}H_{16}O_3$ MW: 184	-	-	17.9

[a] MW = molecular weight.

1.C Spice production and quality

The quality of saffron is largely dependent on the actual and relative concentrations of the 3 main secondary metabolites; crocins, picrocrocin and safranal – and these levels are determined by a combination of cultivation/harvesting practices and post-harvest treatments including stigma separation and drying[*1, 4, 9, 10*].

1.C.1 Horticultural aspects

The countries where saffron is commercially produced exhibit a range of climatic conditions, but a typical climate for successful cultivation of the species may be described as having warm to hot and relatively dry summers with mild autumns and cool to cold winter[*1, 2, 10*]. The plants are frost and snow tolerant (as long as snow cover is not extended) and indeed some degree of winter chilling is beneficial for production probably through initiation of flowers for the next seasons crop[*2*]. Generally, good saffron growing areas have moderate to low rainfall with most of the annual rain falling in the winter months rather than summer when the plants are dormant and more prone to fungal rot diseases[*1*], although exceptions to this pattern are evident such as in Kashmir

where the Indian monsoon may bring rain in summer[*53*]. Lower rainfall areas such as in morocco and parts of Iran require autumn and winter irrigation[*1, 10*] as it is beneficial to flower yield and quality for the corms to receive a significant quantity of water approximately 3 weeks prior to flowering[*1, 2, 5*].

Friable soils with mild pH and good drainage (without high clay content) are considered best for saffron crops (to avoid waterlogging and resulting fungal rot – and allow root penetration) and although some application of fertilizers may be necessary, particularly rich soils are not required. Indeed there is some evidence to suggest that particularly fertile soils with high nitrogen contents, while encouraging vegetative growth, can impair flower yield and quality – and rather it is appropriate ratios of K, N and P and certain micronutrients that gives a good balance between vegetative vigour, flower yield and quality[*3, 5, 10, 54, 55*].

Soil preparation usually involves tillage to a depth of approximately 30 cm and formation of raised beds, commonly in parallel rows, which facilitates better drainage and ease of picking. Corms are usually planted in 2-4 lines (separated by 15-20 cm) per bed, at depths between 10 and 20 cm and with horizontal separation distances of 2-5 cm. Deeper planted corms tend to divide less (into daughter cormlets) resulting in fewer flowers in the years following planting, while more shallow planted corms tend to gradually become semi-emergent at the surface as they expand and multiply which, while resulting in more flowers for a few seasons, eventually reduces yields as this lifting and crowding causes corm degeneration through competition and dehydration[*1, 2, 5, 10*].

Because of this tendency for crowding and the increased probability with time of fungal rot diseases becoming established underground, it is common (and recommended) for growers to lift corms periodically, divide daughter corms off the mothers and replant in fresh (fallow) ground. This period varies greatly among saffron producing regions and may be up to 12 years where growth is slow, however the most common period reported is approximately 4 years[*1, 5, 10*].

It is recommended that during this lifting process corms be treated with an antifungal agent. A variety of fungal diseases are reported to effect *Crocus* crops including species of *Penicillium* and *Fusarium* in Italy[*1*], *Phoma* and *Rhizoctonia* in Greece and elsewhere[*10*] and *Stromatinia gladioli* in Australia[*56*]. This latter disease, studied in Tasmania, indicated pathogenic attack via the roots and recommended lifting and replanting every 3-4 years when a partial de-husking of the corms allowed more effective penetration (and corm survival) when dipped in a solution of fungicide prior replanting.

Alternative indoor methods of cultivation have been reported[*57-59*] including hydroponic systems[*60*] providing improved control of plant growth, onset of flowering, yield of flowers, extension of flowering period and protection of flowers prior to harvest from environmentally caused degradation (such as drying by wind, leaching by rain and photo-degradation) – but this practise is not common and no information is available as to the commercial viability of such a practice where infrastructure costs may be significant.

1.C.2 Harvesting and processing methods

Saffron flowering occurs randomly through a crop over several (4-6) weeks so that flowers need to be harvested on virtually every day over that period with considerable judgement applied to pick flowers at the right stage of maturity. Most sources recommend picking in the early morning to prevent premature drying and photo-degradation during the day. Picking is usually performed by grasping the peduncle or base of the corolla between finger and thumb and either twisting or cutting with the fingernail. As leaves and flowers tend to emerge concurrently, it is important to avoid

excessive damage to the leaves during picking as this may compromise the growth of the corms over the summer and affect the following seasons flowering[*2, 5, 8, 10, 61*].

When rain occurs during the harvest time it is preferable to pick flowers as early as possible when tepals are still suppressed as this partially protects the stigmas from being leached of pigment by water.

It is best to separate stigmas for drying away from strong sunlight and as soon as practical after harvest; and this is usually performed by hand. After this, it is recommended that controlled drying be commenced as soon as possible to minimise chemical degradation[*2, 5, 61*].

While both commercial flower picking and stigma separation are almost universally performed by hand making the crop labour intensive, attempts have been made to facilitate mechanization of these tasks. Trials of mechanical harvest devices are reported from Italian studies conducted in the 1980^{s} by Galigani and others[*61*]. These trials all incorporated vacuum suction mechanisms, with and without cutting actions, and although the devices proved successful in picking some flowers while alleviating the physical strain (of bending) on the pickers, a significant proportion of the flowers were missed and leaves cut, a high proportion of impurities (such as dirt) included, and the operation was no quicker than manual harvest. Furthermore, these studies showed that the suction device had a premature drying effect on the flowers so that spice quality, particularly colour, suffered as a result.

More recently Ruggui and Bertetto have reported the design and trial of a mechanical harvesting device based on a combination of camming and oscillating movements[*62*]. This invention is claimed to selectively cut flower stalks in preference to leaves, despite both being subjected to the forces involved. While this device has potential in that it proved relatively effective in cutting flowers, no significant trial of saffron flower (which were not in season) field harvest, or data on leaf cutting or damage was given. The individual cutting motion also suggests that while its use may prove more user-friendly (in removing the need to bend) no significant saving in time (and thus labour costs) may be gained Moreover, the design incorporated a suction device to collect flowers and no analysis of saffron quality from flowers harvested with the device was available.

Any successful mechanical system would require much more uniform flowering to accomplish significant labour saving or efficiency. This would probably require hormonal and/or environmental manipulation of the plants. While application of gibberellins has been shown to promote flower formation (and auxins to inhibit this)[*63*], no commercial application or field trials has been reported and it is considered by both Ordoudi and Tsimidou (2004) and Souret and Weathers (1999) that not enough is known at present about *Crocus* physiology to achieve adequate uniformity of flowering this way [*10, 64*].

Recent trials of indoor (glasshouse) environmental control of saffron crops has shown that timing of flowering onset can be controlled to a degree and product yields potentially improved through more reliable flowering[*57, 59*], but no improvement in actual flowering uniformity was described. Furthermore, the induction of hysteranthy (the emergence of flowers preceding that of leaves) would be required to prevent significant cutting of leaves that would compromise growth and the yield of ensuing crops. Some environmental control of hysteranthy, involving extended incubation of corms at specific temperatures, has been reported[*64, 65*]; but this would almost certainly require indoor cultivation.

Glasshouse based environmental control may be a more efficient method of producing high yielding quality saffron, but considerably more research would be required to establish the precise methods for this as well the cost benefits given that this form of horticulture is much more capital intensive than the current practises. Even supposing that mechanical harvesting of a uniform flowering crop could be achieved then that would place considerable pressure on the stigma

separation and drying facilities, as the yield of flowers currently processed over several weeks would need to be processed at once.

The Italian trials[*61*] also included testing of various devices for separating stigmas from tepals, stamens and peduncle. These included use of fans with wind tunnels and screens as well as vibrating boards. These were not very successful in that a high proportion of stigmas remained attached to other parts and often the whole corolla would curl even more tightly around the stigmas unless the flower was totally open and beyond the optimal harvest stage such that quality was compromised. The premature drying effect of the air currents also affected later spice quality particularly loss of aroma. Nevertheless, references of industry use of fan based winnowing devices for separation of stigmas from other matter may be found – such as in the review by Ordoudi and Tsimidou[*10*] and on Indian websites[*66, 67*], but no detailed information about mechanisms, effectiveness or spice quality are available. All the saffron produced in India is for the domestic market in that country and does not have a reputation for consistent high quality[*5*].

1.C.3 Saffron drying methods

Crocin and picricrocin levels peak in *Crocus* flowers at full bloom and so assuming that the flowers are harvested then, the main determinant of saffron quality is the drying process where picricrocin is hydrolysed to form safranal[*4, 9, 10, 45*]. The conditions of this drying process including; the temperature, rate of drying, final moisture content and physical air flow characteristics, are all critical to the level of safranal produced and retained in the stigmas as well as any concurrent loss of the colour compounds (crocins) that may occur due to photodegradation and/or enzymatic or thermal hydrolysis[*32, 45, 68, 69*]. In effect, drying tends to have concurrent but inverse effects on the aroma and colour strengths of the spice and so the aim is to maximise the former while minimising the latter.

From the evidence available in the literature there is considerable uncertainty about the ideal conditions for the drying of saffron. Stigmas have traditionally been dried by methods such as sun drying (India and Iran), toasting over hot ashes (Spain) and drying slowly in a darkened room at 30-35°C (Greece)[*1, 9, 32, 45, 68*]. Of these, the Spanish methods have been regarded as producing the best quality of saffron[*9, 15, 33, 69*] and although recent work has provided some detail of the actual conditions involved[*32*], no precise determination of optimal drying conditions has been provided.

The range of recent scientific studies and reviews available provides contradictory information as to the best drying conditions. In reviewing the topic, Cadwallader[*9*] summarized the work of Riana et al[*45*] and others, concluding that a temperature range of 35-45°C without airflow or freeze drying was optimum for good conversion of picrocrocin to safranal without excessive loss of colour. He emphasized that temperatures below this required a long drying period, which resulted in excessive enzymatic degradation of crocins, while excessive temperature resulted in thermal degradation of these pigments. Other studies have given evidence for quality saffron with aroma from drying conditions such as: 80°C for 30 min in an oven[*38*] presumably without strong airflow, 110°C for 2 min and 70°C for 30 min with a strong airflow[*69*]. The traditional Spanish methods involving toasting over hot coals (or some other heat source) were until recently thought to be effecting drying temperatures below 50°C, but have now been shown involve maximum temperatures ranging from 75°C to 121°C for periods of 28-55 min.[*32*].

While the various drying methods cited above give different drying temperatures, nowhere has the actual measurement of stigma temperature during the drying process been reported. This may be

important because the fresh stigmas prior to drying consist of approximately 90% water[*1, 10, 45, 70*] most of which must evaporate during the drying process to achieve a final moisture content of <12%. This must cause an evaporative cooling effect[*71-73*], and therefore, for a significant part of the drying period stigma, temperatures should theoretically be lower than the surrounding air temperature, which is presumably what has been measured in all of the reports above. No studies have given any measurement of the humidity of the drying environment either, something which would impact on both the degree of evaporative cooling and the rate of drying[*74, 75*].

The work of Carmona *et al*[*29, 32, 37*] has highlighted some of the unknowns involved in understanding how saffron aroma is formed and thus in determining optimum drying conditions. Their findings related to further generation of safranal (and other related aroma compounds) in previously dried saffron at high temperature (>>100°C). It indicated that such generation was maximised when the initial drying had been conducted at 70-90°C rather than at more moderate temperatures. They also demonstrated that thermal treatment of crocetin (the nuclear moiety of the crocins) could also result in generation of safranal. This led to their proposition that an alternative pathway of safranal (and other volatiles) production from *trans*-crocins existed. This proposed mechanism, while unproven, has interesting connotations for determination of optimum drying temperature as it suggests that the full potential for release of aroma compounds upon cooking of the spice may only occur if the original drying was at sufficiently high temperature to generate precursors or intermediates in this pathway.

In Australia, the Industry currently uses commercial food dryers with constant non-adjustable airflow at temperatures ranging between 40°C and 55°C. Under these conditions stigmas require drying over a period ranging from 45 to 90 min. to produce saffron with a moisture content of < 12%[*5*]. The use of higher temperatures to produce saffron of purportedly high quality in Spain and the studies of Carmona *et al* (cited above) suggests that there may be potential for greater aroma development under at more elevated temperatures. This should be investigated through careful application of experimental treatments including temperature and airflow, and which should also include measurement of actual stigma temperature and humidity.

1.C.4 Effects of storage

The degradation of saffron pigments (crocins) under various storage conditions has been described by a number of authors[*10, 30, 32, 36, 45, 58, 76, 77*] and the kinetics of this degradation studied in depth[*35, 78*].

The crocins are essentially photo-labile such that exposure to strong light (visible and UV) for even a few hours can have a significant effect on lowering colour strength (half life of ~3 hrs)[*78*]. It therefore follows that exposure to even dim light over months or years in storage will cause significant deterioration[*10, 36, 77, 79*].

Similarly, the effects of temperature and relative humidity are well documented and, if not as dramatic as strong light, may still cause rapid deterioration (see Figure 1-5). Dark storage at 40°C and 75% RH has been shown to cause almost complete loss of crocins within 70 days[*36*]. Despite this, crocins may actually be considered as relatively thermo-stable compounds[*32, 80*] suggesting that such temperature related degradation may be largely enzymatic.

Storage in such conditions also resulted in a 40% reduction in picrocrocin, which suggests an inverse effect that these storage conditions, particularly humidity, may have on aroma. Given sufficient moisture (either from incomplete drying or via storage in high humidity), further enzymatic hydrolysis of picrocrocin to form HCC and safranal may occur, particularly if the

original drying temperature had not been so high as to permanently deactivate the glucosidase (see figure 1). Thus, as the pigments degrade during storage, the aroma strength may gradually develop, a process known as "soft dehydration" that is sometimes used commercially to produce high aroma saffron (eg in Greece)[*1, 29, 68*].

It is however, doubtful as to whether this method of saffron production/storage is optimal as, even with careful temperature control, significant colour loss due to oxidative degradation of crocins (possibly enzymatic) must occur[*10, 35*]; an effect that has been observed in the Australian Industry[*5*]. Furthermore, loss of picrocrocin during "soft dehydration" does not always result in generation of safranal[*29, 45, 81*].

It has been suggested that storage at low temperatures (<35°C) but with intermediate water activity may be a useful compromise, allowing gradual aroma development while limiting colour loss to a moderate rate only[*9, 10*]; and that product stored this way should therefore have a very limited "shelf life" of no more than 1 year[*45*]. However, if good aroma development can be achieved during initial drying, then there is no reason why this storage method (with inevitable colour loss) should be employed. Given sufficient initial drying followed by dark storage at low temperature and humidity in sealed containers, "shelf life" could be much longer than this (3 years at least) as evidenced by a number of studies[*35, 51, 68*].

An analysis of storage effects of Australian product in relation to the type of drying method employed would provide valuable information as to the potential "shelf life" of saffron generally and aid in determining the optimum drying conditions for later storage of the product.

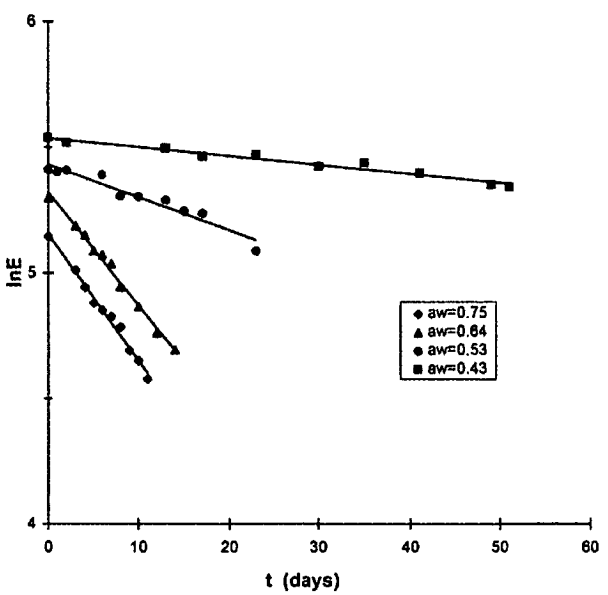


Figure 1-6: Degradation plots for saffron carotenoids (colouring intensity InE at max = 443 nm) following storage at 40 °C in different relative humidity (aw) environments (from Tsimidou and Biliaderis 1997[33]).

1.C.5 Methods of quality measurement

The standard measurement of saffron quality and composition is the ISO-3632 (2003)[*82*] test methods. Amongst these tests the colour, flavour and aroma strengths of saffron are measured by a spectrophotometric analysis method that quantifies these components by simple absorbance values of a filtered diluted aqueous extract of the ground spice. For safranal in particular, the method is

reportedly subject to inaccuracy due to; the lack of solubility of safranal in water, interfering absorbance at 330nm from *cis*-crocins and post extraction degradation of picrocrocin[*33, 68*]. Despite this, the literature lacks definitive evidence of such inaccuracy in the form of direct analytical comparison of the ISO method with methods measuring crocins, picrocrocin and safranal directly.

Various other means have been used for quantifying the important components of saffron including; thermal desorption GC[*33, 83*], HPLC analysis of polar and non polar extracts – or with a polarity range capable of measuring the polar crocins and picrocrocin as well as the non-polar safranal from the one polar solvent extract[*84*] – and GC analysis of solvent[*38, 68, 85*] and supercritical CO2 extracts[*34*]. Not all these methods are readily available to industry and doubt remains as to whether a single extraction can provide an accurate measure of the true relative levels of all these components[*85, 86*].

The literature includes a wide variety of reported safranal concentrations measured by different extraction and analysis methods in saffron of different origins including: approx 800 ppm in Indian saffron extracted with 80% ethanol[*70*], approx 1500 ppm in Spanish saffron by supercritical CO2 extraction[*34*], 1070-3970 ppm in Spanish saffron analysed by thermal desorption/GC[*33*], up to approx. 4000 ppm in Indian saffron by simultaneous hydro-distillation/extraction (SHDE)[*45*], and in Greek saffron up to 1200 ppm when cold extracted with diethyl ether or up to 6400 ppm in the same material by (SHDE)[*68*], a process where significant extra safranal may be generated due to the heating involved.

The total crocins (as the sum of the glycosyl esters of crocetin) contents of commercial samples of saffron have typically been reported from 5% of dry Wt. for sun dried stigmas[*70*] to 17% of dry Wt. for vacuum oven dried samples having poor aroma development[*45*]; although for quality product with good aroma development, values ranging from 6% to 16% of dry Wt.[*45, 49, 70, 76*] have been reported. Picrocrocin levels in commercial saffron and/or fresh stigmas have been reported ranging from <5% up to 18% of dry wt.[*49, 76, 85, 87, 88*].

Given the doubts over the accuracy of the ISO method and the array of other method that have been employed, there is clearly a need to establish a reliable method of component measurement before experimental drying treatments are employed. By providing further evidence of the accuracy, or otherwise, of the which ever methods are tested, particularly in comparison to the ISO method, the objective of gaining consensus in regard to acceptance of an appropriate standard analysis method will be furthered.

1.C.6 Saffron spice extracts

Extraction of saffron spice to obtain products for commercial sale (as opposed to extraction for analysis, culinary or other use by customers) is not common, probably because the source material is too expensive to allow further value adding[*1, 5, 10*]. The potential for producing oleoresin products (as an intended replacement for the actual spice) has been investigated[*89-92*] and mid-polarity solvents were found to be best for extracting a good colour product with reasonable aroma. However, given the great discrepancy in polarity between the colour and aroma components it is doubtful that any product can be produced with sufficient pigment and aroma strength to compare in quality to the actual spice. The potential enzymatic post harvest incubation of saffron to enhance concentration of aroma compounds combined with extraction by mid polarity solvents may yield commercially valuable products, though it would still seem likely that only low quality (reject) spice would be viably used for such production.

It is more likely that saffron extracts may be incorporated into medicinal or healthcare products such as skin lotions due to the potent antioxidant properties of the pigments[*93, 94*] that may be made more attractive by the inclusion of saffron aroma or pigment factors[*18*]; however, this is really just an extension of the normal culinary or dying use of the spice and manufacturers are more likely to purchase actual spice than prepared extracts[*5*].

There has been limited work reported on investigation of alternative extracts from the fresh flowers and/or waste flower parts (after the removal of stigmas for spice production). Intense yellow hexane extracts of waste flowers have been obtained but with a very low yield of absolute (0.18% after removal of waxes)[*92, 95*]. GC-olfactory analysis of such extracts has revealed a strong contribution of honey notes to the overall aroma. Although no chemical identifications were reported from this study, other headspace, distillate and extract analyses of the aroma of fresh flowers and extracts have indicated that major volatile components include phenylethyl alcohol and its acetate, 4-hydroxy-benzene-ethanol, nonanal, hexanal, heptanal, tetracosane, ethyl hexadecanoate and heptadecane; with the first 3 of these being associated with the honey notes[*96*].

No compound identification has been given for the pigments responsible for the colour of these extracts nor has any specific (commercial) use been suggested for them other than possible colouring of textiles[*95*].

More polar (water/methanol/acetonitrile) extractions of saffron spice and fresh tepals and anthers of *Crocus* flowers have revealed the presence of significant amounts of flavonoids including various kaempferol glycosides[*10, 30, 97-102*]. These compounds have been implicated in some of the reported medicinal properties attributed to saffron extracts[*103-106*], but again, no specific commercial products or uses have been reported. The tepals also contain anthocyanin pigments that could be used for dying[*96*], although the concentration of these in *Crocus* is not especially high compared to many other species[*107, 108*].

1.D Bioactive attributes

The following quote from Culpeper's Complete Herbal (A.D. 1653) Reproduced from *The Essential Saffron Companion* by John Humphries (1996)[*8*] gives an illustration of the long history of the traditional medicinal use for saffron.

"*It is an herb of the Sun, and under the Lion. Not above ten grains must be given at one time; a cordial if taken in an immoderate quantity, hurts the heart instead of helping it. It quickens the brain; helps consumptions of the lungs, and difficulty of breathing, it is excellent in epidemical diseases, as pestilence, smallpox, and measles. It is a notably expulsive medicine, and a good remedy in the yellow-jaundice. It is a useful aromatic, of a strong penetrating smell, and a warm, pungent, bitterish taste. It is said to be more cordial, and exhilarating than any of the other aromatics, and is particularly serviceable in disorders of the breast in female obstructions, and hysteric depressions. Saffron is endowed with great virtues, for it refreshes the spirit, and is good against fainting-fits and the palpitation of the heart; it strengthens the stomach, helps digestion, cleans the lungs and is good in hysteric disorders. However, the use of it ought to be moderate and reasonable; for when the dose is too large, it produces a heaviness of the head and sleepiness; some have fallen into an immoderate convulsive laughter, which ended in death. A few grains of this is commonly a dose, though some have prescribed it from half a scruple to a scruple and a half*"

This history (see section 2.A.3) has led, particularly in the last 2 decades, to considerable scientific investigation of the spice's bioactive properties, medicinal applications and the physio-chemical mechanisms involved. In 1971, Hartwell, in reviewing the historical medicinal uses of saffron[*109*] highlighted its use an anti-cancer agent to treat tumours of the liver, spleen, kidney, stomach and uterus; a contribution, together with Basker and Negbi's review in 1983[*110*] and the continued references to saffron in Chinese and Indian medical literature[*12, 111, 112*], that has helped to rekindle modern scientific interest in saffron. The volume of scientific publications, pertaining to these aspects of saffron, has expanded dramatically since the early 1990's.

1.D.1 Anti-cancer properties

This growing body of research has demonstrated that saffron extracts (of the spice and other plant parts), including the constituent metabolites such as crocins, picrocrocin, safranal and flavonoids, possess chemo-preventative properties against cancer[*15, 99, 113-117*]. Such anti-tumour effects of saffron extracts have been demonstrated *in vivo* in mice and rats[*118, 119*] and *in vitro* with human carcinoma cells without having any toxic side effects at the doses used[*16, 120*]. These effects have been reported with a variety cancer cell types including those from melanomas[*117, 119, 121*], leukemia[*122, 123*], lymphomas[*6, 114*], pulmonary adenocarcinoma[*116, 124-126*], colon adenocarcinoma, cervical adenocarcinoma, ovarian sarcoma, hepatic sarcoma, and osteosarcoma[*6, 15*].

Most studies where the effects of saffron components have been differentiated (as opposed to the effect of the extract as a whole), have indicated that most (but not all) of the significant anti-cancer action is due to the pigments; particularly crocin (*trans*-4 crocin) and/or crocetin[*15, 118, 119, 122, 123, 127, 128*]. Safranal, however, has been shown to be anti-mutagenic[*120, 129*] and to have the

most rapid cytotoxic effect where tumour cell necrosis (rather than just inhibition of cancer formation) is a desired component of the treatment[*16*].

Flavonoids and flavonoid glycosides that have been detected in saffron spice and tepals[*30, 100, 101*] may also play a role in the overall anti cancer effect of saffron extracts through enzyme inhibition or their known anti-oxidant/radical scavenging properties[*103, 104*]. Factors with anti tumour/cytotoxic properties such as glycoconjugates and lectin have also been identified in extracts from *Crocus* corms[*130, 131*].

A number of mechanisms of action have been proposed for the anti-cancer properties of the components of saffron. These include inhibitory effects on DNA and RNA synthesis[*125, 132, 133*] and possibly on protein synthesis (although there are contradictory reports on this)[*125, 126, 134*], inhibition of free radical chain reactions through anti-oxidant/radical scavenging properties[*16, 93, 115, 135-139*], cytotoxic effects associated with cellular DNA- protein interaction[*6, 113*], inhibition of early antigen expression in tumour cells[*140*] and modification of the action of various cellular enzymes[*103, 116*]. Saffron extracts have also been shown to ameliorate the negative side effects of other anti-cancer drugs while preserving their anti-tumour activity[*6, 137, 141*].

Abdullaev, one of the chief authorities on this subject indicates that human clinical trials of saffron extracts are proceeding. Though the potential for the use of saffron products in modern cancer treatments is clear, the cost of saffron spice is a currently the most prohibitive factor to its widespread use in medicine[*15, 16*]. In response to this problem, tissue culture studies have been conducted investigating the potential for *in vitro* propagation of stigma like structures from various explant tissues. Such structures have been developed by a number of different techniques[*1, 6, 64*], and although the concentrations of the main components (crocins, picrocrocin and safranal) in most cases were lower than in "normal" saffron[*81, 142, 143*], instances of propagation producing contents roughly equivalent to or higher than "normal" saffron have been reported[*144, 145*]. The most useful application of tissue culture techniques may be in the study of the enzymatic control of the various steps in saffron metabolite synthesis with a view to eventually boosting metabolite production via genetic engineering[*1, 6, 22, 23, 26, 27, 146*].

1.D.2 Other "medicinal" properties

Among the more commonly reported general effects of saffron products are tissue and organ protective properties that are evident in studies on; hypoxia, alcohol or geno-toxin induced neurological ischemia[*139, 147-151*], renal ischemia[*93, 136*] and myocardial ischemia[*152, 153*]. These effects appear to be related to the anti-oxidant and free radical scavenging properties of saffron components[*6, 15, 134*] which are also claimed to aid in wound healing[*154*].

Saffron extracts, and most particularly safranal, have been shown to have anti-inflammatory, analgesic, anti-convulsant[*14, 155, 156*], anti-depressant and anti-anxiety[*157, 158*] effects; including in humans[*159, 160*]. A significant potential has been demonstrated for the promotion of learning and memory in patients with neurodegenerative disorders[*161*] including treatment of Alzhiemers[*158, 162*] and Parkinsons[*151*] diseases.

Hypolipidaemic and anti-coagulant effects, particularly associated with crocetin, have been reported in a number of studies[*128, 136, 163*] and these may be useful in the treatment of atherosclerosis and heart disease[*64*].

The use of saffron extracts in potential skin care products is being investigated by a number of researchers[*89, 92, 94, 95*]. The proposed benefits of such use are supported by work showing that

saffron extracts promote hyaluron formation in skin cells resulting in a significant moisturising/anti-wrinkling effect[*64, 138*].

1.D.3 Implications

The growing body of scientific evidence for the array of medicinal properties possessed by saffron seems likely to promote future demand for the product worldwide. That many of these properties may be attributed to specific components of the spice (such as crocins and safranal) or to components from other parts of *Crocus* flowers points both to the desirability for production methods that maximises the concentration of these components in the spice; and to the need to identify the compounds and establish their concentration in the "waste" flower parts.

1.E Research objectives and experimental design

Notwithstanding any potential that exists for mechanisation of saffron harvesting and processing, these tasks continue to be labour intensive. Given this, relatively minor saffron producing countries with relatively high labour costs – such as Australia – cannot hope to compete purely on a priced based commodity trading basis. Rather, the industry in Australia has identified the need to market their product as being of demonstrably superior quality than that coming from countries with relatively low labour costs; and therefore, to generate demand for their product despite it being considerably more expensive[19]. Optimisation of the concentrations of the main chemicals that give saffron its properties is central to this objective.

This project aims to provide new knowledge that allows the Industry in Australia to better compete against imported product on the local market – and eventually through export of product onto the world market. It is envisaged that this research-based assistance will aid Industry in 2 ways:

- By analysing the character quality parameters of the local product (relative to the international standard and imported products) and then applying experiments to determine how post-harvest processing technology – and in particular the drying process – may be altered to optimise the quality of the saffron product.
- To investigate the potential for new products from the waste flower parts by conducting experimental extractions and chemical analysis of the products. Any commercially viable new products from waste material would aid industry through value adding to each saffron crop.

The various investigative aspects of the project are summarised in figure 1-7.

1.E.1 Post-harvest technology to optimise saffron spice quality

From the preceding review of the literature it is apparent that – while there are traditional conceptions of the best way to process and dry saffron (which vary from region to region) and some incomplete and inconclusive scientific data exists on the matter – there is no definitive method and/or set of drying conditions that has been shown to optimise saffron quality. Indeed, there is still much to be learnt about the chemical processes involved in the formation and/or retention of the important chemical components in saffron.

In attempting to determine what the optimum drying conditions might be with the intention of applying them to Australian production of saffron, it is first necessary to determine what the quality of the local product is and how this correlates with the methods currently used.

The major focuses of this part of the project are thus:

- To apply the ISO-3632 (2003)[*82*] test procedures to Australian saffron from a range of growers to establish whether the product meets the ISO standard parameters, to assess the range of quality being produced and to compare this to the imported product from overseas competitors.

- To develop and trial analytical methods designed to measure the actual levels of the important secondary metabolites in Crocus stigmas and dried product and to compare these methods to the ISO test measurements (which only give relative absorbance estimates) to determine the best means of quantifying quality parameters.
- To conduct experiments designed to investigate alternative drying procedures to the current method employed by Tas-Saff and in so doing establish optimum post harvest treatment conditions for the production of saffron. Factors for investigation include: drying temperature, actual stigma temperature, duration and rate of drying, airflow, humidity, effects on stigma physical structure and post-drying heat treatment.
- Once optimum conditions have been determined, to then adapt the drying apparatus/technology to best incorporate these parameters and to test the new methods on a commercial scale during a harvest period.

1.E.2 Potential for products from waste flowers

The production of saffron from *Crocus* flowers utilises < 5% of the dry Wt of the flowers picked. The preceding review of the literature reveals that while there have been some reports of saffron flower extract products with attractive properties and that other compounds with potential uses such as flavonoids may be present in the flowers, the information on the chemical contents of the *Crocus* tepals and stamens is scant. The fresh flowers are known to have a delicate but pleasant aroma.

Preliminary steam distillation and solvent extraction trials will be undertaken with waste petals and the products subjected to analysis including organoleptic, GC/MS and HPLC/MS techniques to determine the chemical composition of such products as an indication of their potential for commercial use.

1.E.3 Summary

The Australian saffron producing industry is very small and (for the reasons discussed earlier) vulnerable to competition from major world producers. Given the burgeoning interest in saffron for its medicinal properties/uses it is reasonable to expect that domestic and global demand for saffron spice and other saffron products may grow significantly in coming years. By giving the local industry the means to better compete through demonstrable optimisation of quality and possible introduction of a new "value adding" product, Australian growers will be better placed to take advantage of such demand.

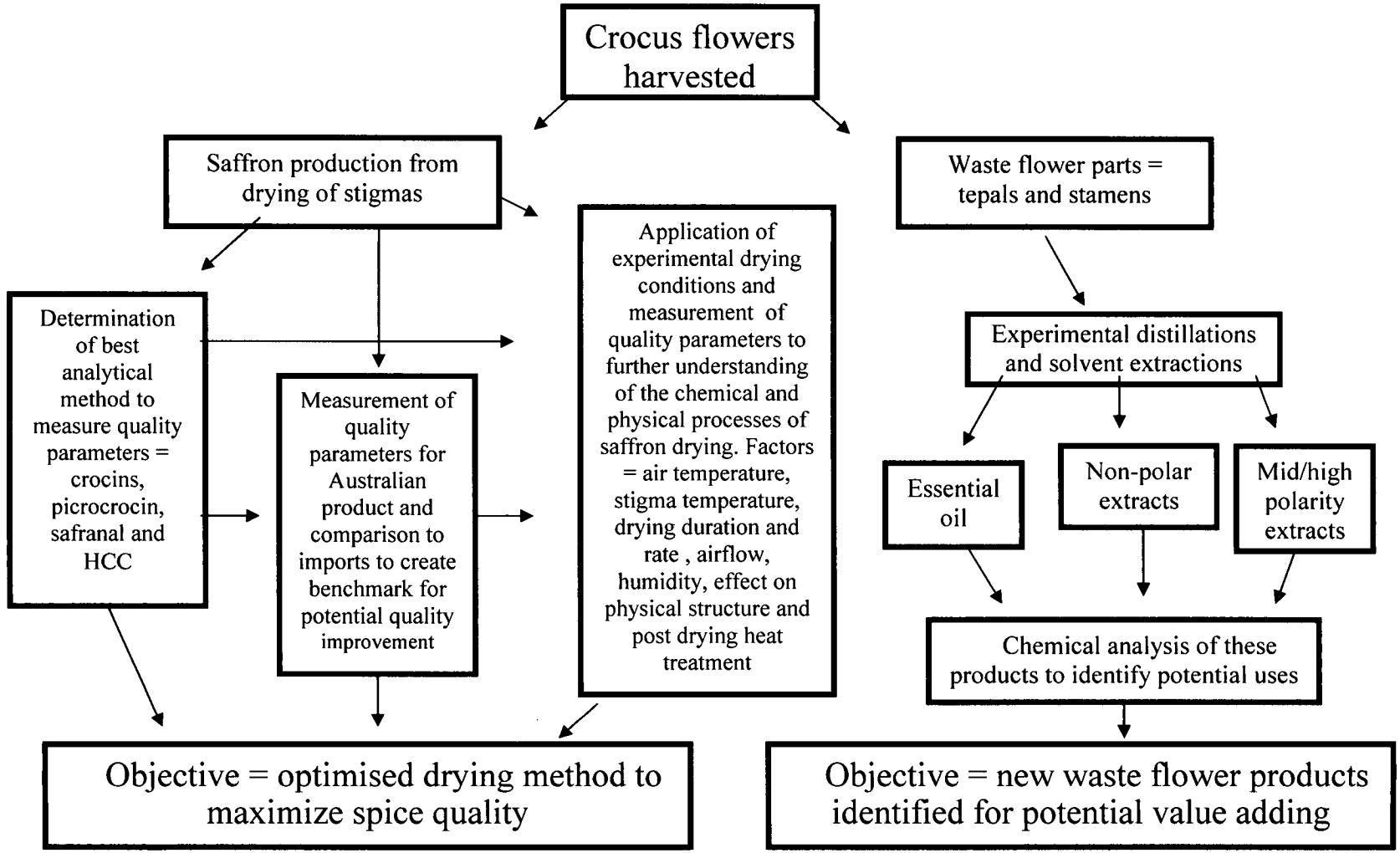


Figure 1-7: Inter-relational representation of different aspects of project including objectives.

2 GENERAL METHODOLOGY

2.A Saffron analysis

Where specific technology or procedures have been applied to experiments or particular processes, they are described in the relevant experimental sections of the results chapters.

2.A.1 Collection of Stigma Material

The stigmas used for each distinct experiment were collected from the same farm (or crop) on the same day and used for the experimental drying treatments within the next 20 hours. Flowers were harvested between 8 and 10 am, except were otherwise indicated. Flowers were only harvested where sufficient peduncle elongation rendered picking convenient, whether or not the tepals had opened.

The flowers were sealed in plastic containers and placed in a cooled "esky" maintained at 3 - 6°C for transport to the laboratory. Once there, the flowers were stored at 5 °C until immediately prior to application of drying treatments when stigmas were separated from tepals and stamens by incising the filaments at the pistil.

The exceptions to this immediate application of drying treatments were those stigmas frozen as part of the treatment or for later enzyme and enzyme inhibitor experiments, which were stored in sealed vials at -10 °C until use.

2.A.2 ISO analysis protocol

All measurements described in this report as ISO testing procedures were performed according to the International Standards Organization (ISO) Technical Specification (TS) 3632-2 Test methods 2003, reference number ISO/TS 3632-2:2003(E)[*82*]. That part of the standard analysis pertaining to measurement of colour, bitterness and aroma strengths is detailed as follows:

Samples of 2 g saffron (weighed to 4 decimal places) and ground with a mortar and pestle (for a standard 1 minute) before being passed through a 500 μm sieve. A sub-sample was taken for dry weight determination before a 500 mg (weighed to 4 decimal places) sample of powder was suspended in 900 ml of double distilled water in a 1 L volumetric flask containing a magnetic bar. This suspension was stirred on a magnetic stirrer in a dark room for 1 hour before the bar was removed and the volume made up to 1 L. After thorough mixing, 2 x 25 ml aliquots were taken and made up to 250 ml in volumetric flasks. These solutions were filtered through # 2 Whatman filter paper in a Buchman funnel before measurement of the absorbances at 440, 360 and 257 nm using quartz cells in a Shimadzu spectrophotometer. The colour, bitterness and aroma strengths are expressed as numbers derived from the formula; AU/\wt. of saffron powder in kg.

2.A.3 Dry weight determinations

All saffron, fresh stigma and waste flower dry weight determinations were performed according to the ISO protocol (ISO/TS 3632-2:2003(E)[*82*]). This involved weighing of the samples (to 4 decimal places) in open aluminium containers followed by drying in an oven at 104 °C for 16 hours. Upon removal from the oven the containers were sealed with a lid, allowed to cool to room temperature for 10 minutes and reweighed to allow determination of % dry weight content from the weight loss measured. It should be noted that in each case this weight loss would have included a small proportion of volatile compounds from drying at this temperature; something acknowledged in the ISO protocol.

2.A.4 Saffron extractions

For the determination of the pigments, picrocrocin and comparison of safranal extraction, a polar solvent was required and as for other commonly used methods, methanol was chosen [*30, 32, 76, 84, 164*]. Additionally, for comparison of safranal (and HCC) extraction a relatively non-polar solvent was required[*38, 68*] and hexane was used in this case.

After treatment, samples were reweighed then divided up with a 4 stigma subsample of each replicate taken, combined with those from the other replicates of the same treatment for dry weight determination. The remaining stigmas of each replicate were split into 2 subsamples of approximately 8 stigmas, which were each, weighed (to 4 decimal places at 20°C) into separate sealed vials. To each of these treatment replicates, 5 ml of HPLC grade hexane and 0.492 mg of β-cyclocitral (Fluka, 95%) (as an internal standard), were added and the vials sealed. To the remaining sub-sample from each replicate, 5 ml of HPLC grade methanol was added and the vial sealed.

The extraction method used was adapted from the ultrasound assisted method used by Kanakis *et al[68]*as this did not employ heat which could cause compositional changes. Extraction samples were subjected to ultrasonification for 1 hour in the dark before being left on a shaker at 100 rpm for 20 hours in the dark at 15°C. The samples were then allowed to settle out in the dark for 1 hr. before an aliquot of 1 ml of each of the extracts was transferred to 2 ml GC vials and sealed. These samples were then stored in a freezer at -10°C before analysis.

2.A.5 Gas chromatography (GC)

A Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionisation detector (FID), a split injection system and a HP-1 cross linked methyl silicon gum column (30m length, 0.32 mm id, 0.33 mm film thickness) was used. Injections of 5 μl were made with an injector temperature of 250 °C. Carrier gas was N2 at a column flow of 1.8 ml/min., a head pressure of 8 psi and a split ratio of 1:50. The oven temperature program was 50 °C for 2 min. before rising at 9 °C/min to 290 °C and held for 11.3 min. The detector temperature was 295 °C.

In order to accurately quantify the response of safranal by GC-FID, calibrations giving determination (re standard curves) of the response of 8 concentrations of safranal (Fluka, 75%, cat. No.17306) between 26 and 500 mg/ml, in both hexane and methanol, were run. The determination of HCC was made relative to the response of safranal in hexane, as this compound is structurally very similar. After it was decided to adopt the hexane extraction as the standard method for quantification of safranal and HCC, this calibration was repeated with internal standards (C13 =

tridecane and C18 = octadecane) added to determine response coefficients relative to these standards. These coefficients were then used to quantify safranal and HCC in all later samples free from any changes in the response of the GC.

2.A.6 High performance liquid chromatography (HPLC)

A Waters HPLC coupled to a Waters 996 Photo-Diode Array detector was used. Trans-4-crocin, trans-3-crocin, cis-4-crocin, trans-2-crocin, cis-3-crocin and trans-2-crocin were measured at 440 nm and picrocrocin was measured at 250 nm. Separation and identification of these compounds was made with reference to the methods and results reported previously by of Castellar *et al* [164]and Lozano *et al.* A Waters Nova-Pak C18 column (3.9 mm x 150 mm) and an Alltech Econosphere 5 micron C18 guard cartridge at a flow rate of 1 ml/minute were used. Solvent A was methanol and solvent B was 1% acetic acid in Milli-Q water. The gradient was 20%-A to 80%-A at 45 minutes, and then to 100%-A at 45.01 minutes and this was held to 52 minutes. Re-equilibration between runs was 10 minutes. The known molar absorptivities of crocins and picrocrocin (in water and alcohol) were used to calculate concentrations of these compounds in the extracts, using the known conversion factor between molar absorptivity and raw PDA peak area at this flow rate (for this system) based on a β-carotene standard. The molar absorptivities were those stated by Casteller *et al*[*164*] or calculated from the extinction coefficients (E1%/1 cm) at each wavelength, given by Davies[*165*]. For crocin (used for all the crocins which possess the same chromophore) this was e440 = 133750 M-1 cm-1 while for picrocrocin, e 250 = 10100 M-1 cm-1 and for β-carotene the E1%/1 cm at 440 nm = 2620 and thus e440 = 140432 M-1 cm-1.

2.A.7 Mass spectroscopy (MS)

Samples were analysed by GC-MS to confirm the safranal and HCC identities using a Varian 3800 GC coupled directly to a Varian 1200L triple quadrupole mass spectrometer. A Varian Factor-Four VF5-MS (25m x 0.25 mm x 0.25 micron film) with helium as carrier gas at a flow rate of 1.2 ml/min was used. Aliquots of 2 µl were injected into a Varian 1177 injector using the split mode (15:1) at a temperature of 210°C, and the column oven was held at 6°C for one minute and then ramped to 14°C at 5 degrees per minute to 140 C and then to 28°C at 20 degrees per minute. The m/z range from 35 to 350 was scanned every 0.3 seconds. A reference spectrum of HCC was available from an in-house specialized terpene library. Other identifications were based on either this source or the NIST library or published MS spectra from saffron analyses[*29, 31, 39-41, 43, 44, 51, 68, 97, 166-168*]. Confirmation of identifications was made by comparison with Kovats indices reported in these sources.

2.A.8 Statistical analysis

Linear regression analyses of the standard curve plots of safranal GC/FID response against tridecane and octadecane internal standards and for comparison of extraction and analysis methods were performed using Microsoft Excel 2003. Statistical analyses of component contents for comparisons of treatments were carried out using the procedures of the SAS statistical package,

version 9.1, 2003, SAS Institute Inc., N.C. USA. This included a log transformation of the safranal and HCC data sets before analysis of variants (ANOVA) as this provided a closer fit to a normal distribution. Significance in these analyses was determined at the 0.05% level while error bars on all graphs represent standard deviations.

2.B Waste flower studies

2.B.1 Collection and drying of waste flowers

Flowers used were part of the normal harvest for both production of commercial saffron and for drying experiments. Flowers collected were from ≈ 30 harvest days over 2 seasons. After separation of stigmas (commercially or in the laboratory), the tepals and stamens were either; dried at ambient temperature, taken to the laboratory fresh for non-ambient drying/incubation treatment prior to distillation/extraction, or frozen for later processing.

Ambient air-drying was conducted on each farm by spreading the tepals and stamens over a horizontally suspended net inside a room away from sunlight. Drying was assessed as complete when upon handling, the tepals no longer tended to roll up and adhere one to another. Once dried, they were then taken to the laboratory and stored in the dark at 5 °C until distillation or extraction.

Of the fresh flower parts transported (at 3-6 °C) to the laboratory, those that had already had stigmas removed (as part of the normal commercial harvest) were searched for missed or rejected stigmas so that the mean content of these in the commercial harvesting could be estimated. The flowers were then either, distilled or extracted fresh or after non-ambient incubation/drying treatments conducted in an oven (without airflow), or frozen at –10 °C for later processing.

2.B.2 Extractions

Flower samples were distilled in cohobation units comprising 18 litre stainless steel vats fitted with glass Liebig condensers. Due to the low volume yield of volatiles, the product was collected into 15 ml hexane traps. The sample was recovered from this by first drying the hexane of any residual water with sodium sulphate before filtering through #4 Whatman filter paper and drying off the hexane in an RVE at 30°C.

Solvents used for extractions including hexane, petroleum ether (hexane/pentane 20/80), ethanol and chloroform were all AR grade, redistilled to ensure purity. Extraction of flower samples were made using an initial wash of solvent (at 6 times v/w), subjected to ultrasound for 1 hour and then left on a shaker at 100 rpm in the dark overnight. The solvent was then collected and the flowers washed with 2 further washes of 1 hour each with shaking. The combined solvent was then filtered through glass wool to remove any particulates and dried down in a RVE at 30°C. All procedures during and after extraction of flowers were preformed in very low light conditions to minimize pigment degradation and isomerization.

Conversion of extracts (concretes) to absolutes was achieved by first dissolving the extract in 5 times v/w of warm (50°C) ethanol. This solution was then gradually cooled to 4°C with gentle agitation and bore chilling to –10°C and filtering through a pre cooled Buchner apparatus using #4

Whatman filter paper to remove precipitated waxes. The ethanol was then dried off at 30°C in a RVE.

2.B.3 Saponification of extracts

Extracts were re-dissolved in diethyl ether (0.5 g/100 ml) and mixed with 5 ml of methanolic KOH (30% w/v). This was shaken for 1 hour then left to stand in the dark overnight. The mixture was washed three times with water in a separating funnel and the ether layer dried with 1 g of anhydrous sodium sulphate before being evaporated to dryness in a RVE and redissolved in methanol/hexane (80/20) for HPLC analysis.

2.B.4 Gas chromatographic analysis (GC)

A Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionisation detector (FID), a split injection system and a HP-1 cross linked methyl silicon gum column (30 m C 0.32 mm id, 0.33 mm film thickness) was used. Injections of 2 µl were made with an injector temperature of 250°C in either splitless mode with purging resuming after 2 min. or split at a ratio of 50:1. Carrier gas was N2 at a column flow of 1.8 ml/min., a head pressure of 8 psi. The oven temperature program was 50°C for 2 min. before rising at 9°C/min to 300°C and held for 10.3 min. The detector temperature was 310°C.

2.B.5 High performance liquid chromatography (HPLC)

A Waters HPLC coupled to a Waters 996 Photo-Diode Array detector was used with samples run on either a Waters Novapak C18 (3.9x150 mm) or a Waters Bondapak C18 (3.9x300 mm) column. All flow rates were 0.8 ml/min but mobile phase and gradients varied with the type of samples run. For unsaponified hexane extracts the mobile phase and gradient consisted of 100% methanol for 8 min changing to 80/20% methanol/hexane over 143 min. and held for 40 min.

For saponified hexane extracts (for separation of free carotenoid isomers) a mobile phase of 40/60% water/methanol for 10 min. then graded to 100% methanol over 50 min. and was held for 30 minutes. To achieve best resolution of isomers of free carotenoid (from saponification) this gradient was applied with 2 Waters Bondapak C18 (3.9x300 mm) columns connected in series. Quantification of carotenoids was made by reference to the response of a lutein standard (95% purity obtained from Sigma-Aldrich).

For methanol extracts (for flavonoid analysis) a mobile phase of 2% aqueous acetic acid/methanol at 95/5% graded to 65/35 over 15 min. and then to 100% methanol by 25 min. and held for 5 min. Detection was achieved at 330 nm.

Quantification of flavonoids was made by reference to a flavonoid mixture (kaempferol glucosides) of known concentrations obtained from Paula Andrade[*169*].

2.B.6 Mass spectroscopy (MS)

Samples were analysed by GC-MS using a Varian 3800 GC coupled directly to a Varian 1200L triple quadrupole mass spectrometer. A Varian Factor-Four VF5-MS (25m x 0.25 mm x 0.25 micron film) with helium as carrier gas at a flow rate of 1.2 ml/min was used. Aliquots of 2 µl of a solution of ≈ 10 mg/ml extract in hexane were injected into a Varian 1177 injector using the split mode (15:1) at a temperature of 210°C, and the column oven was held at 6°C for one minute and then ramped to 14°C at 5 degrees per minute to 140 C and then to 28°C at 20 degrees per minute. The m/z range from 35 to 350 was scanned every 0.3 seconds. Identifications were made on the basis of comparisons with the NIST library, reference spectrums available from an in-house specialized terpene library or published MS spectra from saffron analyses[*29, 31, 39-41, 43, 44, 51, 68, 97, 166-168*].

LC-MS/MS analysis was performed using A Finnigan LCQ operated in the APCI mode with data-dependent MS/MS scans for the most intense ion.

3 OPTIMISING SAFFRON QUALITY THROUGH POST HARVEST TECHNOLOGY

3.A Analytical methodology and assessment of Australian saffron quality and storage

3.A.1 Application of ISO analysis method

Experimental

The full range of ISO testing procedures[*82*] were applied to 3 samples of local product from the 2003 season that had been judged by the Industry Partners to be of sufficient quality for sale. These were compared to the results for a competitor's imported product that had the most widespread vendor distribution in Australia.

Results and discussion

While the results (**see table 3.1**) of these tests showed that for the physical characteristics (ISO clauses 5-12) all the samples met the highest quality standard, for the compositional tests of bitterness and colour strength, the imported product was well below this standard. The Australian samples all met this standard, though T2 was on the threshold for colour. While these results might serve as an indication that the locally produced saffron (which was an unknown mixture of product from different growers) compares well to the product with which it is competing, a much more comprehensive survey of quality was needed to determine the degree of variability between samples from individual growers and between different imported products.

Of the tests indicated in **table 3.1**, clauses 5, 6, 7, 10, 11 & 14[*82*] essentially serve as indications of contamination (intentional or otherwise) of saffron product rather than being indications of actual quality as affected by the drying process. Clause 14 is a test for chemical contamination such as from intentional spiking of saffron with artificial dyes to give false colouring strengths. Given that no problem was indicated by these tests in this initial sampling of locally produced saffron it was decided to exclude these clauses from further sampling of a more extensive range of local grower samples. Ensuing analyses would therefore include clause 13 (aroma, colour and bitterness strengths) as well as % moisture (clause 8) and crushing and sieving (clause 9) as these were judged to be significantly affected by the degree and method of drying.

Table 3-1: Full ISO Test Procedure Results for Australian and Imported Saffron

ISO Test Method	Characteristic	Sample	Sample	Sample	Sample	ISO Requirement
		T 1	T 2	T 3	Imported Product	
ISO 3632-2 clause 5	Identification Test	Samples identified as only from *Crocus sativus*	Samples identified as only from *Crocus sativus*	Samples identified as only from *Crocus sativus*	Samples identified as only from *Crocus sativus*	No other vegetable matter to be found other than from *Crocus sativus* Linnaeus.
ISO 3632-2 clause 6	Floral Waste Content	0.13%	0.06%	0.11%	0.37%	Category Extra 1 = < 0.5%
ISO 3632-2 clause 7	Extraneous Matter Content	0.02%	0.01%	0.01%	0.01%	Category Extra 1 = < 0.1%
ISO 3632-2 clause 9	Moisture and Volatile Matter Content	11.7%	13.9%	11.41%	15.8%	< 12%
ISO 3632-2 clause 10	Total Ash Content	5.01%	4.90%	4.75%	4.67%	< 8%
ISO 3632-2 clause 11	Acid Insoluble Ash Content	0.29%	1.11%	0.39%	0.27%	Categories 1&2 = < 1.0% Categories 3&4 = < 1.5%
ISO 3632-2 clause 12	Crushing and Sieving	0.98%	0.96%	0.95%	0.97%	More than 95% of the crushed material passes through 500 mm sieve
ISO 3632-2 clause 13	Bitterness (picricrocin) Absorbance Reading $E^{1\%}$(1cm, 257nm)	92	92	90	67	For Category 1 = > 70
ISO 3632-2 clause 13	Fragrance (safranal) Absorbance Reading $E^{1\%}$(1cm, 257nm)	28	28	24	36	All Categories = 20-50
ISO 3632-2 clause 13	Colour (crocins) Absorbance Reading $E^{1\%}$(1cm, 257nm)	198	190	230	147	For Category 1 = > 190
ISO 3632-2 clause 14	Identification of Main Characteristics (Thin-layer Chromatography)	No extraneous bands evident	No extraneous bands evident	No extraneous bands evident	No extraneous bands evident but 2 of the pigment bands very faint	Observation of chromatogram in daylight and UV light shows only bands for main saffron components

3.A.2 Comparison of ISO and alternative analysis methods

Experimental

In order to provide a measure of the relative accuracies of the ISO standard methods (for aroma, bitterness and colour strength) in comparison to extraction and analytical measurement of safranal, picrocrocin and total crocins, a range of individual grower and imported samples collected between 2004 and 2006 were analysed in both ways. To this end, absorbances of aqueous solutions of samples were measured at 257, 330 and 440 nm according to the ISO clause 13 test procedures (for aroma, colour and bitterness strengths)[*82*]. Samples were also extracted with hexane and analysed by GC/FID for safranal content; and extracted with methanol and analysed by HPLC/UV-Vis for picrocrocin and total crocins content (see chapter 2 for details). The sample set (n=125) for the safranal/aroma strength comparison was much larger than the other 2 comparisons (n=30) as safranal measurement was included as a routine part of the analysis of other experiments (see chapter 3.A.3&4); the results from which were included in this comparison.

Results and discussion

A typical GC-FID chromatogram of one of the hexane extracts is shown in **Figure 3-1a** where peak detection had been confirmed by comparison with known standards and by GC-MS with reference to the NIST database. **Figure 3-1b** shows a typical HPLC profile with absorbance detection at 440 nm (for crocins determination) and 257 nm (for picrocrocin determination)

The plot of ISO aroma strength (absorbance at 330 nm) against safranal content (**figure 3-2**) clearly shows a very poor correlation (r^2 = 0.1311 for linear regression) between the 2 types of measurement. The inaccuracy of the ISO method as a measure of aroma strength (due to the safranal content) has been referred to by a number of authors[*28, 33, 68, 69*], citing the combination of insolubility of safranal in aqueous extracts, absorbance interference by *cis*-crocins at 330 nm and post extraction degradation of picrocrocin as reasons. This inaccuracy has not previously been statistically demonstrated by concurrent comparison of the ISO measurement with actual safranal content, but is now confirmed by result shown in figure 3.1. The ISO method is clearly not a meaningful measurement of aroma strength.

The comparisons of the ISO measurements of bitterness and colour (absorbances at 257 and 440 nm) with the actual picrocrocin and total crocin contents are given in **figures 3-3 and 3-4** and show much closer correlations from linear regressions despite the much smaller samples sets (than for aroma). While this shows that ISO method for bitterness and colour is a reasonable estimation of these relative strengths, Carmona *et al*[*28, 32*] have suggested that structural variation in saffron due to different drying conditions may lead to differences in extraction efficiency of aqueous solutions. This may result in significant variation (or error) in the ISO method which specifies only a 1 hour extraction time whereas the methanolic extractions for HPLC analysis of samples was conducted over 21 hours to allow complete dissolution.

For consistent determination of aroma, bitterness and colour qualities in all the ensuing experiments it was decided to only use the analytical measurement of actual safranal, picrocrocin and total crocins.

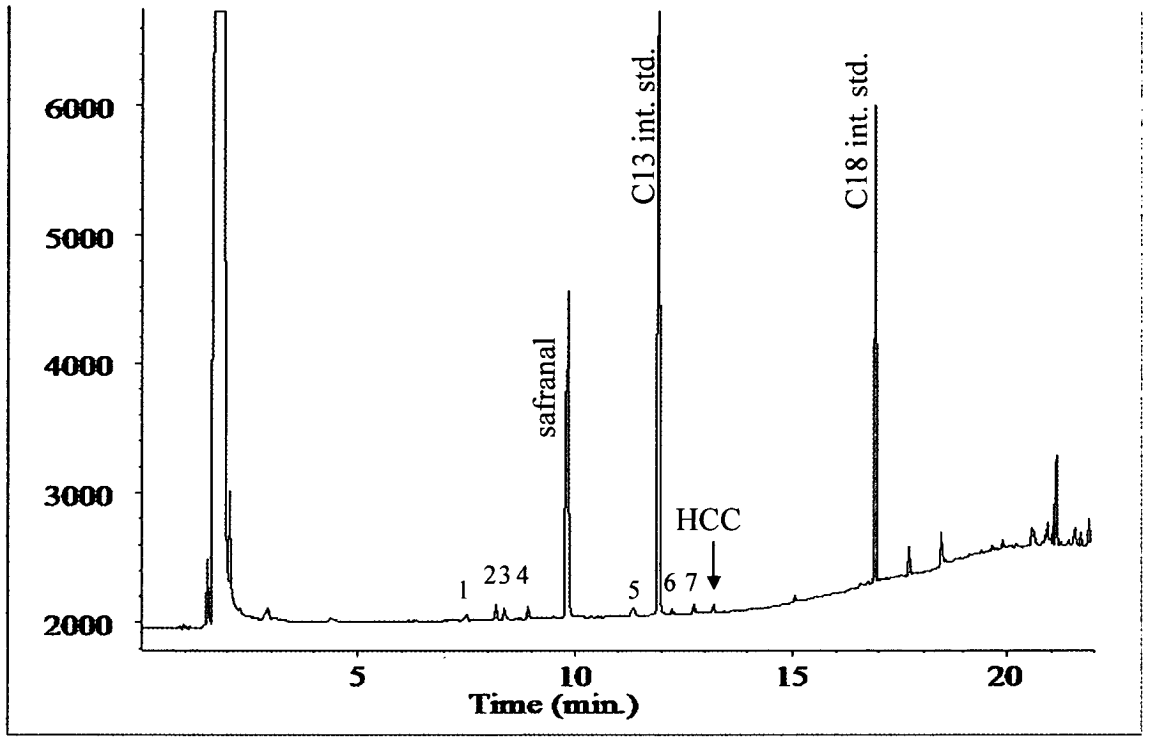


Figure 3-1a: Gas Chromatogram of typical hexane extract of saffron showing safranal and HCC peaks with internal standards tridecane (C13) and octadecane (C18). Other minor components include: 1 = linalool, 2 = isomer of safranal, 3 = 2-phenylethanol and isophorone, 4 = isomer of safranal, 5 = 2-phenylethyl acetate, 6 = 4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one & 7 = 4-hydroxy-2,6,6-trimethyl-3-oxo-1-cyclohexene-1-carboxaldehyde (see General Methodology-Chapter 2 for published MS data used for identifications)

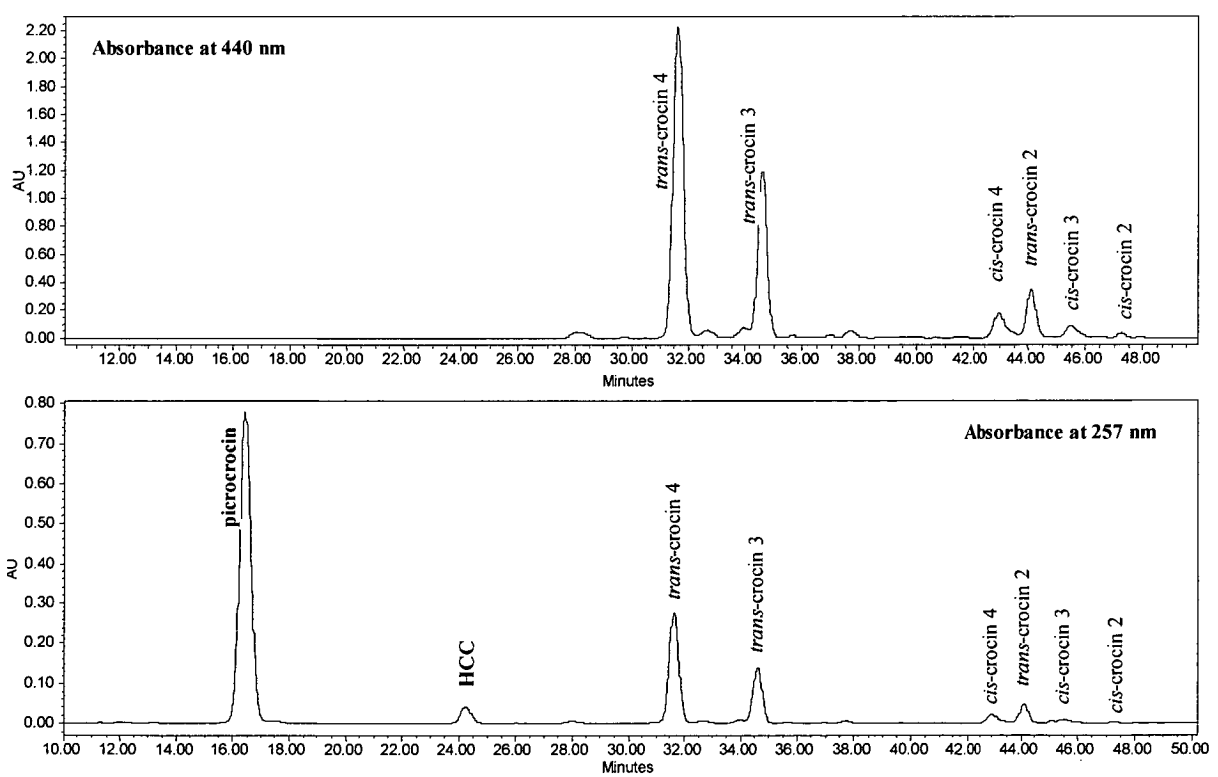


Figure 3-1b: Typical HPLC-/UV-Vis chromatograph of a methanol extract of saffron.

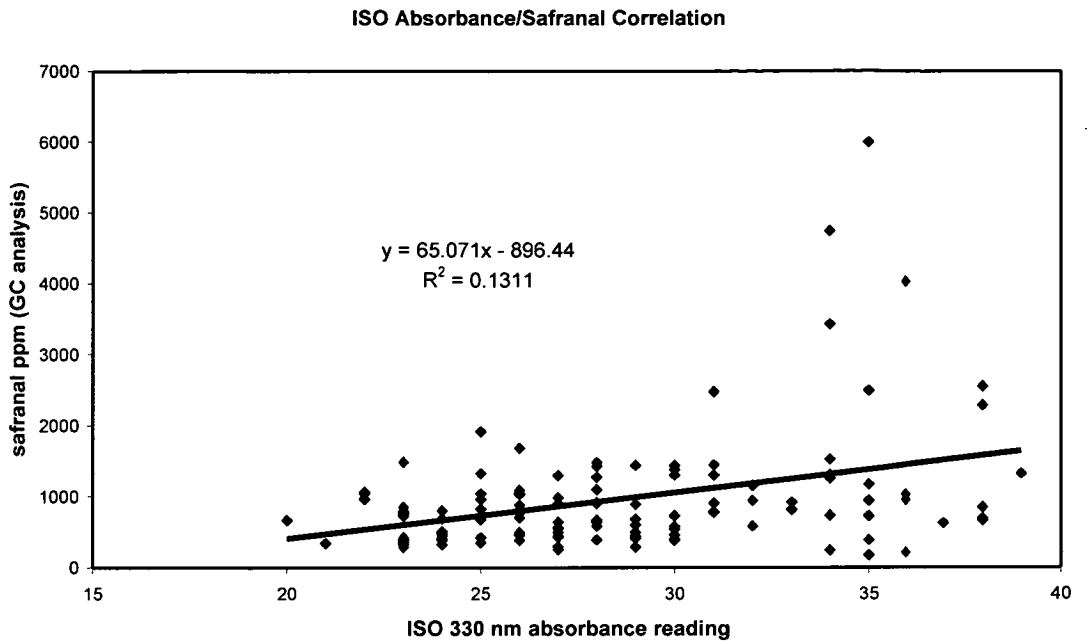


Figure 3-2: Correlation of ISO measurement of aroma strength and safranal content from GC analysis

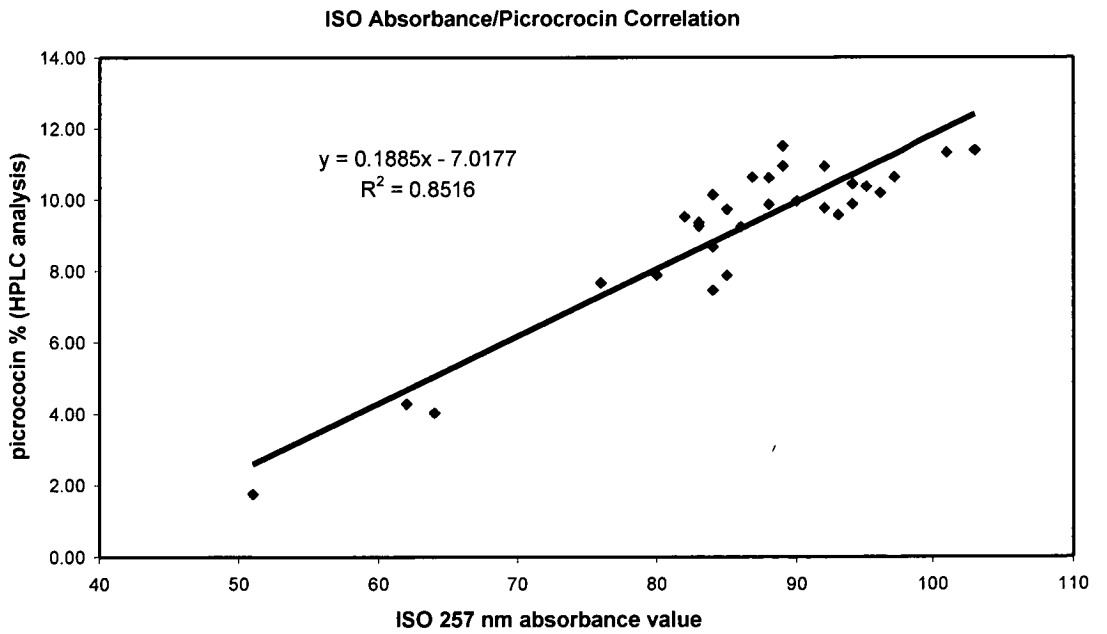


Figure 3-3: Correlation of ISO measurement of bitterness strength and picrocrocin content from HPLC analysis

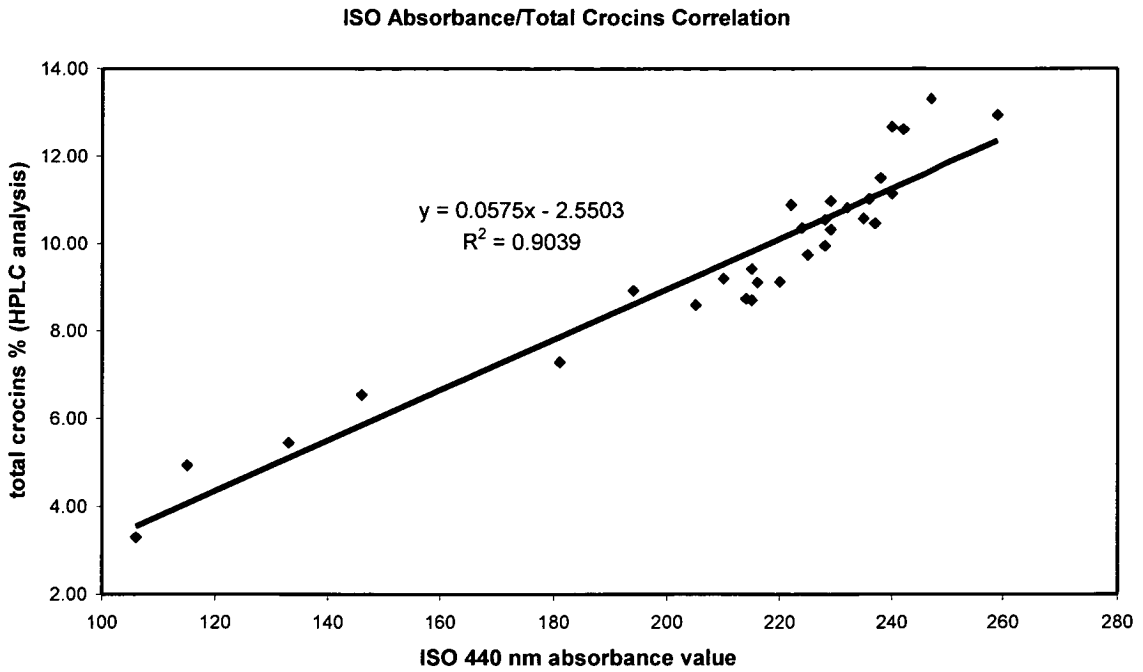


Figure 3-4 Correlation of ISO measurement of colouring strength and total crocins content from HPLC analysis

3.A.3 Comparison of local and international (imported) product

Experimental

In light of the results above (3.A.1), it was decided to survey a range of local samples from individual growers from harvests from seasons 2004-2007 inclusive. Each grower had dried their own saffron using a standard procedure prescribed by the Tas-Saff directors that employed an apparatus that utilises heated airflow at temperatures between 35-50°. Testing of the individual grower samples provided an indication of the quality consistency of the application of this procedure. A number of commercial samples of saffron product either imported or obtained privately from overseas were supplied by Tas-Saff and these were tested along with the grower samples for comparison purposes. All saffron was stored in sealed jars in the dark at ambient temperatures. It should be noted that the time between actual drying of the samples and analysis was not consistent as it varied from a few days up to several weeks.

For this survey the testing procedures were limited to the measurements of bitterness, colour and aroma strength as well as the % moisture as these are directly affected by the drying process. The other tests serve as indications of contamination (intentional or otherwise) of saffron product rather than being indications of actual quality as affected by the drying process. Hexane extraction and GC analysis (as described in chapter 2) of sub-samples of each saffron product was also carried out as an alternative measure of aroma strength for determination of safranal contents.

Results and discussion

From the survey of saffron samples from individual growers produced between 2004 and 2007(**table 3-2**) it is apparent that a significant number of samples were not sufficiently dried according to the ISO requirement of no more than 12% moisture. Over the 4 year period 44% of samples had moisture contents exceeding this. This under-drying was still apparent (18% of samples) from the 2007 season despite growers being warned in each of the preceding years of this potential problem.

Over the 4 year period 6 % of samples had bitterness strength absorbances below the ISO requirement, while some 15% of samples were deficient for colour strength. All the samples fell within the ISO aroma strength absorbance range, but as previously demonstrated this measurement may be considered too inaccurate to be useful (see 3.A.2) while there is no set requirement for actual safranal content.

In some cases the measurement of high moisture was accompanied by reduced bitterness and colour and relatively high safranal contents such as samples TS-7, TS-15 and TS-20 while in others, such as TS-8, TS-14 and TS-17 this was not so. Given that the time elapsing between drying and analysis of samples varied between a few days and several weeks the correlation of moisture content with post drying chemical changes is therefore not apparent. The storage of the samples over a significantly longer period would be required to test any such correlation.

The analysis of imported samples in **table 3-3**, while certainly not a comprehensive survey of saffron production quality from around the world, gives an indication that the problem of high moisture content from either insufficient drying or subsequent re-absorption of moisture is common to other saffron producers resulting in degradation of colour contents. This colour loss may be a result of either insufficient drying or photo-degradation either in storage or prior to drying. It could also be due to the actual conditions during drying.

There has been an assumption perpetuated in the literature that production of saffron with high aroma development (measured as safranal content) requires either a very slow drying process or post-drying enzymatic activity, both of which also result in loss of colour (crocins)[*6, 9, 45*]. In regard to this it is important to note is the analysis of import I which shows that it is possible to produce saffron with a high safranal content that has retained a high colour strength. This sample had a relatively low moisture content suggesting that post-drying enzymatic production had not been the source this safranal.

Table 3-2: Selected ISO analysis parameters for Australian saffron samples from individual growers produced between 2004 and 2007. (TS = Tas-Saff)

Sample	Year produced	ISO 3632-2 clause 9 (moisture and volatile content) %	ISO 3632-2 clause 13 (Bitterness) Absorbance E1% (1cm,257nm)	ISO 3632-2 clause 13 (Colour) Absorbance E1% (1cm,440nm)	ISO 3632-2 clause 13 (Aroma) Absorbance E1% (1cm,330nm)	Safranal ppm in saffron (dry Wt) by GC
ISO Requirement		< 12%	Absorbance at 257 nm = > 70	Absorbance at 440 nm = > 190	Absorbance at 330 nm = 20 - 50	Not specified
TS-1	2004	11.7	92	198	28	916
TS-2	2004	13.9	92	190	28	644
TS-3	2004	11.4	90	230	24	394
TS-4	2004	14.8	85	225	26	496
TS-5	2004	17.5	75	205	27	552
TS-6	2004	14.2	91	230	26	386
TS-7a	2004	20.6	69	182	34	1534
TS-7b	2004	15.6	38	83	38	2289
TS-8	2004	18.3	90	236	23	295
TS-9	2004	17.9	76	200	23	387
TS-10	2004	16.1	78	179	32	589
TS-11	2005	16.2	89	208	30	399
TS-12	2005	14.4	86	222	23	327
TS-13	2005	15.2	74	212	27	297
TS-14	2005	17.7	91	248	28	590
TS-15	2005	16.5	76	130	38	852
TS-16	2005	14.5	86	227	24	515
TS-17	2005	18.2	83	217	25	431
TS-18	2005	13.6	85	230	24	508
TS-19	2005	16.5	80	196	21	349
TS-20	2005	15.7	82	177	28	907
TS-21	2005	13.5	82	232	29	499
TS-22	2006	14.9	90	218	25	719
TS-23	2006	9.3	92	225	23	401
TS-24	2006	15.3	92	219	25	831

Sample	Year produced	ISO 3632-2 clause 9 (moisture and volatile content) %	ISO 3632-2 clause 13 (Bitterness) Absorbance E1% (1cm,257nm)	ISO 3632-2 clause 13 (Colour) Absorbance E1% (1cm,440nm)	ISO 3632-2 clause 13 (Aroma) Absorbance E1% (1cm,330nm)	Safranal ppm in saffron (dry Wt) by GC
ISO Requirement		< 12%	Absorbance at 257 nm = > 70	Absorbance at 440 nm = > 190	Absorbance at 330 nm = 20 - 50	Not specified
TS-25	2006	9.2	95	224	29	446
TS-26	2006	9.0	103	231	24	409
TS-27	2006	7.9	86	226	29	292
TS-28	2006	9.8	88	220	30	584
TS-29	2006	11.8	76	225	33	925
TS-30	2006	9.3	87	216	27	448
TS-31	2006	12.0	77	164	34	741
TS-32	2006	14.6	90	220	26	1038
TS-33	2006	11.4	96	242	23	733
TS-34	2006	16.1	85	229	26	1095
TS-35	2006	10.7	96	237	23	779
TS-36	2006	11.5	91	229	28	1278
TS-37	2006	12.6	92	236	30	388
TS-38	2006	10.1	85	248	22	1073
TS-39	2006	17.4	101	203	23	1492
TS-40	2006	10.9	66	174	36	217
TS-41	2007	7.6	80	194	26	773
TS-42	2007	14.2	89	227	26	883
TS-43	2007	11.4	88	222	23	857
TS-44	2007	10.6	86	220	27	903
TS-45	2007	13.7	89	227	25	829
TS-46	2007	12.0	85	215	22	971
TS-47	2007	10.1	88	228	25	1048
TS-48	2007	8.2	92	224	23	757
TS-49	2007	13.8	83	214	25	969
TS-50	2007	10.2	82	214	26	1686
TS-51	2007	9.0	96	250	24	331
TS-52	2007	10.8	76	205	26	1050

Sample	Year produced	ISO 3632-2 clause 9 (moisture and volatile content) %	ISO 3632-2 clause 13 (Bitterness) Absorbance E1% (1cm,257nm)	ISO 3632-2 clause 13 (Colour) Absorbance E1% (1cm,440nm)	ISO 3632-2 clause 13 (Aroma) Absorbance E1% (1cm,330nm)	Safranal ppm in saffron (dry Wt) by GC
ISO Requirement		< 12%	Absorbance at 257 nm = > 70	Absorbance at 440 nm = > 190	Absorbance at 330 nm = 20 - 50	Not specified
TS-53	2007	11.4	83	219	25	1331
TS-54	2007	8.8	92	215	24	696
TS-55	2007	7.7	97	225	27	565
TS-56	2007	6.4	101	259	24	457
TS-57	2007	13.6	84	228	27	648
TS-58	2007	11.0	85	217	24	483
TS-59	2007	13.3	84	210	20	674
TS-60	2007	8.9	94	235	27	432
TS-61	2007	9.4	95	236	23	802
TS-62	2007	7.8	94	248	28	1435
TS-63	2007	8.8	103	245	23	759
TS-64	2007	9.0	93	215	30	467
TS-65	2007	9.9	84	181	34	1316
TS-66	2007	10.3	64	133	36	957
TS-67	2007	10.8	87	189	23	733
TS-68	2007	11.6	64	141	35	178
Experimental A	2006	9.6	87	217	25	1922
Experimental B	2007	9.1	80	249	35	5997

Table 3-3: Selected ISO analysis parameters for Imported saffron samples

Sample and Origin	Year acquired	ISO 3632-2 clause 9 (moisture and volatile content) %	ISO 3632-2 clause 13 (Bitterness) Absorbance E1% (1cm,257nm)	ISO 3632-2 clause 13 (Colour) Absorbance E1% (1cm,440nm)	ISO 3632-2 clause 13 (Aroma) Absorbance E1% (1cm,330nm)	Safranal ppm in saffron (dry Wt) by GC
ISO Requirement		< 12%	Absorbance at 257 nm = > 70	Absorbance at 440 nm = > 190	Absorbance at 330 nm = 20 - 50	Not specified
Import A (Iran)	2004	17.7	72	181	35	395
Import B (Iran)	2004	19.7	82	196	38	695
Import C (Iran)	2004	20.3	71	152	37	629
Import D (Iran)	2004	19.6	71	169	35	732
Import E (Iran)	2004	19.8	72	183	35	1181
Import F (Iran)	2006	12.5	65	142	38	2556
Import G (Iran)	2007	8.3	75	207	31	792
Import H (Italian Powder)	2005	12.0	70	154	36	1036
Import I (Spanish Castilla-La Mancha)	2005	7.7	104	263	34	3439
Import J (Spanish Mancha superior)	2007	12.0	62	146	27	498
Import K (Greece)	2007	9.2	51	106	38	668
Import L (Indian)	2008	11.6	79	214	35	949

3.A.4 Storage effects

Experimental

Of the samples analysed above, all those produced from the years 2004-2006 were stored in sealed jars in the dark at 5 °C for 2 years before being re-analysed using the same procedures as in 3.A.3.

Results

From analysis results are shown in **table 3-4**, it is apparent that significant changes had occurred in many of the samples, particularly those with relatively high initial moistures contents (see **table 3-3**). These changes were correlated with initial moistures by plotting this against % colour and bitterness strength changes (**figure 3-5**) and against ISO aroma strength and safranal changes (**figure 3-6**). Both plots have 2nd order polynomial curve regressions shown as the best available description of the correlations apparent.

It is clear that significant picrocrocin and crocin degradation and safranal production occurs where saffron samples are stored with moisture above approximately 12%. This is consistent with the ISO requirement and with the studies of Raina *et al*[*45*] and Tsimidou *et al*[*35, 77*] on crocin degradation in saffron powders and with the work of D'Auria *et al*[*51*] on qualitative analysis of saffron aroma. However these studies had tended to focus on the effect of the actual storage conditions rather than the initial moisture content.

The use of "soft hydrolysis" via the enzymatic pathway for production of high aroma/safranal spice is clearly problematic as the process risks unacceptable loss of colour.

The correlation of initial moisture with ISO aroma strength changes is very poor but is to be expected given the confirmation of the inaccuracy of this measurement and its unsuitability as a measure of aroma/safranal.

Table 3-4: Re-measurement of selected ISO analysis parameters for Australian saffron samples (see table 4-2) after 3 years storage.

Sample	Year produced	ISO 3632-2 clause 9 (moisture and volatile content) %	ISO 3632-2 clause 13 (Bitterness) Absorbance E1% (1cm,257nm)	ISO 3632-2 clause 13 (Colour) Absorbance E1% (1cm,440nm)	ISO 3632-2 clause 13 (Aroma) Absorbance E1% (1cm,330nm)	Safranal ppm in saffron (dry Wt) by GC
ISO Requirement		< 12%	Absorbance at 257 nm = > 70	Absorbance at 440 nm = > 190	Absorbance at 330 nm = 20 - 50	Not specified
TS-1	2004	11.4	85	190	29	895
TS-2	2004	12.6	83	173	33	822
TS-3	2004	11.8	87	225	23	427
TS-4	2004	13.5	65	167	30	738
TS-5	2004	15.6	39	81	34	1264
TS-6	2004	13.0	76	186	29	686
TS-7	2004	16.3	18	46	35	2498
TS-8	2004	15.9	25	48	28	1484
TS-9	2004	15.4	25	61	31	1307
TS-10	2004	15.1	47	136	32	1156
TS-11	2005	15.0	48	105	31	914
TS-12	2005	12.5	66	192	28	674
TS-13	2005	14.3	52	141	30	732
TS-14	2005	14.8	31	99	30	1382
TS-15	2005	15.0	40	88	39	1325
TS-16	2005	13.5	70	190	26	708
TS-17	2005	15.6	21	58	31	1448
TS-18	2005	12.0	72	200	25	680
TS-19	2005	13.9	39	117	27	898
TS-20	2005	14.0	55	127	30	1442
TS-21	2005	12.8	74	214	29	608
TS-22	2006	12.8	65	180	27	988
TS-23	2006	9.9	93	216	23	355
TS-24	2006	13.7	72	177	28	1104
TS-25	2006	9.5	93	231	28	395

Sample	Year produced	ISO 3632-2 clause 9 (moisture and volatile content) %	ISO 3632-2 clause 13 (Bitterness) Absorbance E1% (1cm,257nm)	ISO 3632-2 clause 13 (Colour) Absorbance E1% (1cm,440nm)	ISO 3632-2 clause 13 (Aroma) Absorbance E1% (1cm,330nm)	Safranal ppm in saffron (dry Wt) by GC
ISO Requirement		< 12%	Absorbance at 257 nm = > 70	Absorbance at 440 nm = > 190	Absorbance at 330 nm = 20 - 50	Not specified
TS-26	2006	9.8	101	234	25	356
TS-27	2006	9.0	88	230	27	256
TS-28	2006	9.6	85	204	30	545
TS-29	2006	11.1	72	207	32	950
TS-30	2006	9.8	88	210	26	463
TS-31	2006	11.2	75	149	31	780
TS-32	2006	12.3	77	178	30	1309
TS-33	2006	10.9	93	229	26	817
TS-34	2006	12.9	57	141	29	1445
TS-35	2006	10.5	95	239	24	808
TS-36	2006	11.1	87	210	27	1301
TS-37	2006	11.7	89	218	29	416
TS-38	2006	10.5	80	239	22	1049
TS-39	2006	14.5	48	93	31	2483
TS-40	2006	11.0	65	159	34	249

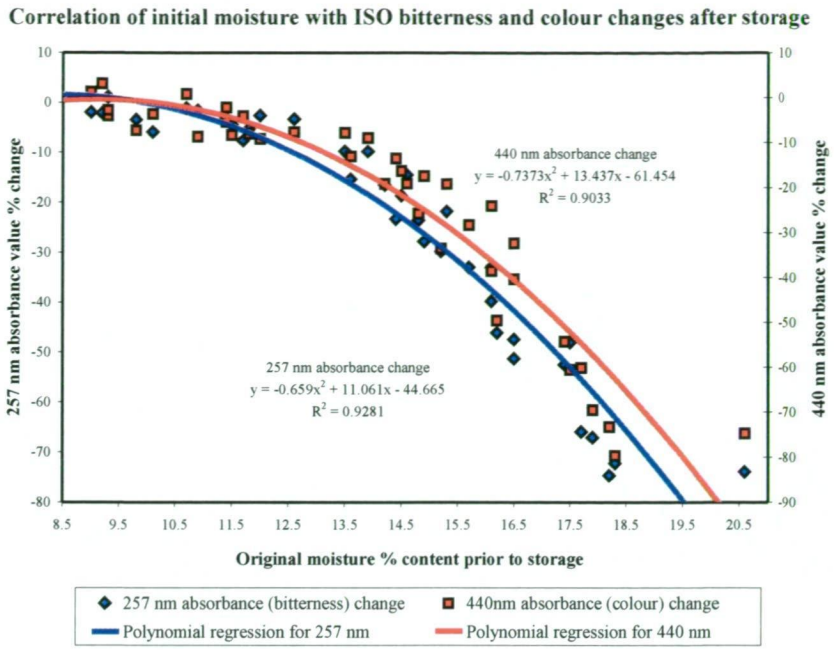


Figure 3-5: Correlation of initial moisture with % ISO bitterness and colour strength changes after 2 years storage.

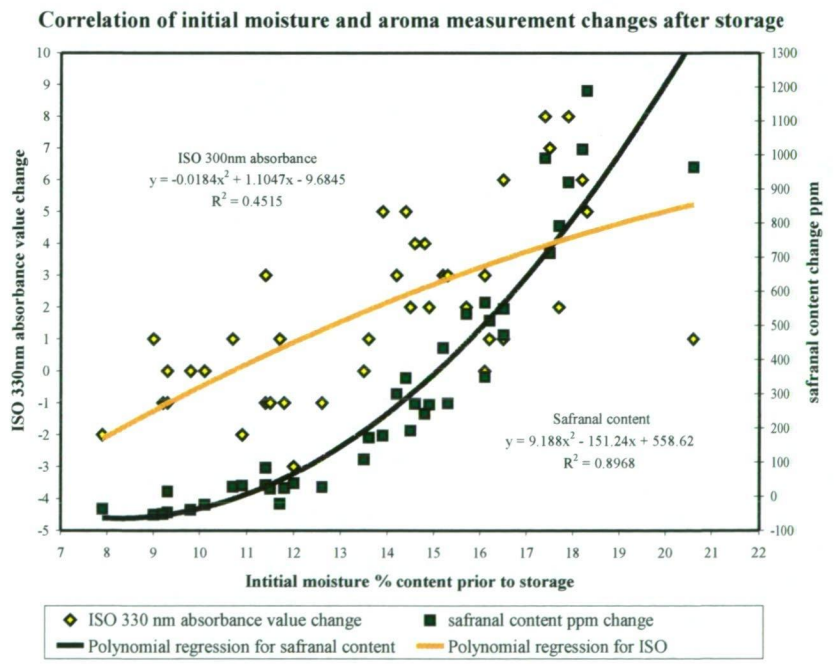


Figure 3-6: Correlation of initial moisture content with ISO aroma strength measurement and safranal content (from GC analysis) changes after 2 years storage.

3.B Initial comparative drying experiments

Introduction

A preliminary comparison of the relatively low temperature drying method currently used by the Australian Saffron Industry (utilising food dryers with fan forced air at between 30 °C and 50 °C) and alternative methods involving higher temperatures was undertaken to test the hypothesis that more safranal could be generated at higher temperatures. Additionally, the concentrations of the other principle components in saffron; crocins, picrocrocin and HCC (the intermediate in the enzymatic pathway for safranal formation) were measured.

Experimental

In order to compare the current drying method to other higher temperature drying methods, the exact operating conditions of the food dryers (employed by local growers) were first characterised. The airflow was measured by the use of a hand held digital wind-speed meter, both inside and over the exhaust vent of the dryers, while temperatures were measured by the use of digital probe thermometers (measuring maximum and mean temperature over set time periods) placed at the same level (second from the top of a stack of 4) of the dryers as experimental drying was performed each time.

Stigmas were collected on 3 distinct harvest dates (as described in the General Methodology 2.A.1). They were either dried with airflow in a food dryer at the temperature setting used for local commercial production, or the same dryer at a higher temperature setting, or in an oven with the fan disconnected at both; moderate temperatures (approximating that used commercially), and at elevated temperatures. Fresh and frozen undried and frozen then dried treatments were also included. The specific conditions of each of these treatments are detailed in **Table 3-5**.

Hexane and methanol extractions and GC and HPLC/UV analyses of the saffron samples from these treatments were performed as described in General Methodology (2.A).

A larger number (approximately 100) of stigmas were collected and dried for both treatments J and K, so that ISO tests (for colour, bitterness and aroma strength)[*82*] could be performed as well as the solvent extractions and moisture determinations. These were also compared to 3 samples dried by the Industry partners with their normal food dryer; one at the normal temperature setting of 40 °C and the other two at the higher settings of 50 °C and 60 °C. It is important to note that while these stigmas were from the same farm they were not harvested on the same day as treatments J and K.

Results and Discussion

The airflow of the commercial food dryer (illustrated in **figure 3-7**) was measured at 2.5 m/s at the exhaust vent; there being no variable control over this speed. The air speed over the stigmas on the stacked trays was found to be 1.8 ± 0.2 m/s.

The temperature settings of the food dryer used were found to be inaccurate in that the 40 °C setting was measured at 46 °C, the 50 °C setting at 58 °C and the 60 °C setting at 72 °C. These results prompted the industry partners to check the temperatures of the dryers

(apparently identical in make and model) used by contracted growers. Their finding was that the devices exhibited various degrees of inaccuracy both above and below the stated setting.

Table 3-5: Description of drying treatments conducted for preliminary experiments and results of safranal extraction methods.

Treatment code	Harvest date	Drying treatment type	Drying Temperature and duration	Airflow	*Safranal yield (ppm) hexane extraction	*Safranal yield (ppm) methanol extraction
A	1	Fresh/no drying	Not dried	None	318 (c)	58 (a)
B	1	Food dryer	46°C for 60 min.	Yes = 2.9m/s	61 (a)	43 (a)
C	1	Oven	43°C for 100 min.	None	93 (b)	47 (a)
D	1	Oven	80°C for 20 min. then 43°C for 70 min	None	920 (d)	54 (a)
E	2	Frozen/ no drying	Not dried	None	518 (g)	55 (b)
F	2	Food dryer	58°C for 20 min. then 46°C for 40 min.	Yes = 2.9m/s	105 (e)	53 (b)
G	2	Oven	87°C for 20 min. then 43°C for 70 min.	None	1596 (h)	80 (c)
H	2	Oven	75°C for 20 min. then 43°C for 70 min.	None	376 (g)	74 (c)
I	2	Frozen, thawed and then dried	87°C for 20 min then 43°C for 70 min.	None	199 (f)	76 (c)
J	3	Oven	43°C for 100 min.	None	377 (i)	73 (d)
K	3	Oven	92°C for 20 min. then 43°C for 70 min.	None	1106 (j)	77 (d)

NB. Means within extraction method annotated by the same letter in parentheses are not significantly different at the P≥ 0.05% level (statistical comparison only made within each harvest time).
* Yield values are all calculated on a ppm/ dry weight of saffron basis

The comparison of the safranal contents from hexane extracts with those from methanol (in **table 3-5)** indicates large discrepancies between the methods. The large differences (up to 25 x) between drying treatments apparent from the hexane extractions correlated very poorly (R2 = 0.19) with the methanol extract results. This evidence confirms the non-suitability of polar extraction for measuring saffron safranal levels indicated by a number of authors[*33, 68, 69*]. It is suggested that this effect may be in part due to poor partitioning between the lipid based membrane tissues and the polar solvent, while methanol will also extract picrocrocin which may undergo post-extraction conversion to safranal leading erroneous measurement of safranal[*38*].

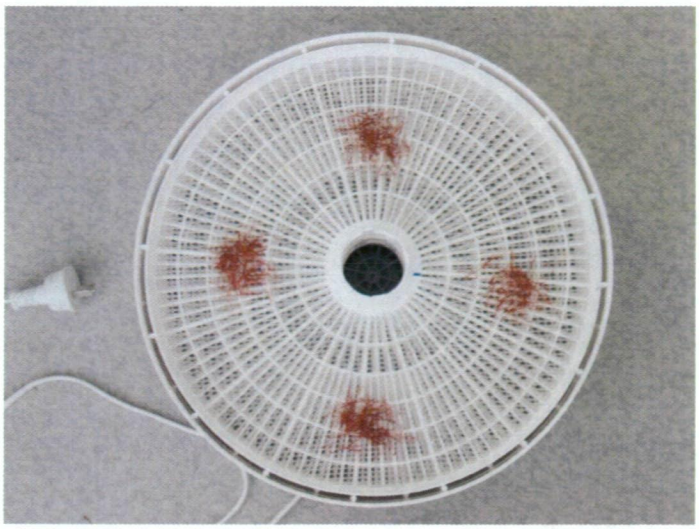


Figure 3-7: Overhead view of commercial food dryer with lid removed showing saffron replicate samples.

The comparison of relative (hexane extracted) safranal and HCC as well as the (methanol extracted) total crocins and picrocrocin contents of the saffron obtained from the different drying treatments is made for each respective harvest date in (**figures 3-(8-10)**. All of the drying treatments (except A and E which were not dried) produced saffron with a final moisture contents approximately at or below the recommended maximum (12%) required by the ISO-3632 standard.

Effect of Harvest Time

Technical constraints limited the number of flowers and thus treatments that could be performed on each harvest day and though it was not the intention of this study to investigate the effect of harvest timing, treatments C and J were identical other than for harvest date (**figures 3-8 & 3-10**). The safranal and HCC contents significantly differed between these suggesting such an effect had occurred, though the factor(s) responsible for this are unclear. Variables such as weather conditions on or before the day of harvest and even relatively small differences in the intervals between flower picking, stigma removal and drying treatment, could have been responsible as much as any physiological effect of harvest timing. For this reason all the following comparisons are only made within each harvest time where the flowers used for the treatments were taken randomly from the total picked that day.

Effect of Temperature

The results (**figures 3-(8-10)**) clearly show that the higher temperature oven dried unfrozen treatments (D, G & K) caused significantly greater conversion to and/or retention of safranal in the saffron than all other treatments at the respective harvest times. Such highly significant increases in the relative safranal contents obtained here (up to 25 times saffron dried at lower temperatures) are most likely due to direct thermal conversion of picrocrocin (see **figure 1-2**) at these high temperatures (80-92 °C), as opposed to the enzymatic conversion pathway via HCC. Enzymes, such as β-glucosidases, although variable in their temperature characteristics, are generally known in plants to undergo thermal inactivation at temperatures above 60°C and become completely denatured at 80°C[*170, 171*]. The fact that these treatments (D, G, & K) also exhibited equal or significantly better retention of crocin pigments at each time in comparison to the lower temperature drying treatments (especially those employing the food dryer, B and F), indicates that this type of drying would be a means for producing quality

saffron with increased aroma. The optimal temperature and duration of this temperature is not determined from these results, and although it would appear to be between 80°C and 92°C, as the highest safranal content was achieved at 87°C, this is not proven because of the different harvest times.

Although the safranal yields from these high temperature treatments were less than some of those reported for quality saffron such as 3970 ppm in Spanish product[*33*]; the work of Kanakis *et al*[*68*] suggests that use of further solvent washes could increase this by 2-3 fold resulting in a safranal content comparable to all but those results reported from methods involving distillation where heating would have caused further conversion of picrocrocin to safranal[*45, 68*]. Moreover, with the levels of picrocrocin detected in saffron samples in this study being 20 or more times the molar equivalent of the highest levels of safranal detected, significant potential for further safranal generation is suggested.

These results appear to contradict both the conclusions of Raina *et al*[*45*] that temperatures of 35-45°C are optimal, and the results of Pardo *et al*[*69*] who used airflow with high temperature treatments, but measured aroma by only the ISO-3632 method. The latter however, also undertook organoleptic comparison that did support the use of higher temperature treatments, and they acknowledged the possible error inherent in the ISO method. These results are consistent with the findings of Loskutov *et al*[*38*], although the safranal increases they reported by drying at 80°C were only 3-4 times that of saffron dried at lower temperatures compared to up to 25 times in this study. The use of more polar extraction solvents (acetonitrile and ethanol) in their study suggests that their determination may have involved an underestimation of the safranal content due to poor extraction.

The higher levels of safranal in the 2 undried treatments (A and E) than in all but the high temperature treatments at each respective harvest time, is explained by continued enzymatic activity occurring in the stigmas during the extraction process as the enzymes would not have been denatured by heat and the stigma tissue would have had sufficient water content for activity to occur. The second step of this conversion pathway, the dehydration of HCC to form safranal, would not have been favoured in the absence of drying, although some of this conversion would have been driven by product removal as safranal partitioned into the organic phase. This conversion would have been further promoted by the tissue disruption caused by freezing and thawing in treatment E, bringing substrates and enzymes together and the much high level of HCC in treatment E further supports this explanation.

The very low level of HCC found in treatment I, despite it being frozen and thawed prior to drying, may be explained by the glucosidase being denatured at the high temperature of initial drying (87°C) with concurrent thermal degradation of any HCC present producing compounds other than safranal; possibly isophorone related oxidation products[*9, 30*]. This suggests that the dehydration step from HCC to safranal in this pathway may be favoured only by drying at moderate temperatures as indicated by Cadwallader[*9*] and certainly the safranal content of the saffron from this treatment was much lower than in G which was identical except for the freezing. Why there was not more safranal produced in the samples from treatment I is not fully explained however, as there was still a significant pool of picrocrocin available for conversion to safranal via direct thermal dehydration (**figure 3-9**). A better understanding of the kinetics of this reaction process, possibly in relation to water activity or changes in the cellular and subcellular structure with drying[*32*], may provide this understanding.

The total crocin contents of treatments B and F (which used the food dryer) were also significantly reduced in comparison to all other treatments. There is no obvious reason to attribute these lower crocin levels to the effect of airflow, whereas the temperature of these treatments (46°C and 58°C) would have allowed greater enzymatic activity than the oven treatments at each respective harvest time; treatment C being below this temperature range,

while D, G, H and I were at temperatures above where thermal denaturing would occur. This explanation is supported by the work of Tsimidou and Biliaderis[*35*] who demonstrated the temperature dependence of crocin degradation between 25°C and 60°C.

The loss of crocins occurring in the higher temperature treatments would presumably be the result of non-enzymatic thermal degradation[*9*], but it would appear that by keeping the high temperature period relatively short this loss was minimized and no enzymatic degradation would have occurred at the subsequent lower temperature as theses enzymes were denatured. In this respect the process is comparable to the way in which many food products are blanched to preserve colour, with such treatments involving brief temperatures above 60°C (typically 70-90°C); a range in which enzymes such as polyphenoloxidases, responsible for browning or colour loss, are significantly and irreversibly denatured[*172-176*].

Effect of Airflow

Significant cross airflow (treatments B and F) in the drying process at temperatures up to 58°C may be deleterious to the quality of the end product as these treatments exhibited the significantly lowest safranal and pigment levels at each harvest time (**figures 3-(8&9)**). This effect however, is not clearly separated from other effects due to the different apparatus used (dryer versus oven) and the temperature differences between them. However, the picrocrocin levels were significantly lower in these treatments than in all others at each harvest time, but without elevated HCC concentrations suggesting that considerable conversion to safranal had occurred but that the product was lost. It is suggested that this loss resulted from evaporation as the headspace was continually exchanged with airflow. This seems consistent with the work of Carmona *et al.*[*32*] where Spanish saffron, dried at high temperatures by the traditional method of toasting over vine shoot charcoal, had aromatic and colour strengths higher than saffron dried in hot airflow at similar temperatures. The interpretation that the lower crocins levels in treatments B and F (**figures 3-(8&9)**) is the result of enzymatic activity being favoured at these temperatures seems likely, although the results of Carmona *et al.* suggest that some direct effect of airflow is possible. They proposed that this might be a factor of dehydration rate and its effect on porosity and thus crocin extractability.

Implications

The results from the preliminary experiments, while clearly showing that improvements in saffron drying methodology could be made with the use of higher temperatures, possibly without airflow, highlighted the need to apply more focussed work to properly isolate each factor and effect. This was particularly so with the use of different rates of airflow at each specific temperature used (i.e. with the same apparatus) to eliminate the effect of harvest time and to control temperatures more accurately to properly determine the optimum temperature and duration of drying. This latter requirement was considered particularly important as the ovens used in these experiments did not have good thermostat control and had a great deal of thermal inertia when heating up and thus great difficulty was encountered in setting them at the desired temperature even though the actual temperatures reached where measured accurately.

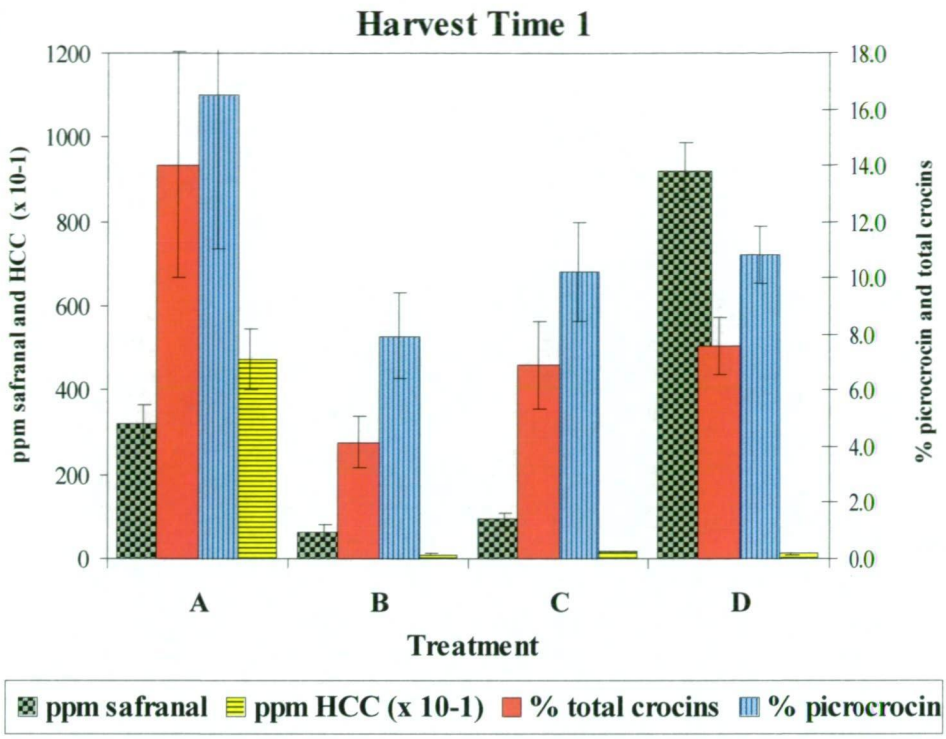


Figure 3-8: Drying treatments at harvest time 1 (see table 3-5 for treatment parameters). NB. While safranal and HCC contents are both given as ppm in this graph, the concentration of HCC plotted is reduced by a factor of 10 such that the highest level of HCC in this plot is approximately 10 X that of the highest level of safranal.

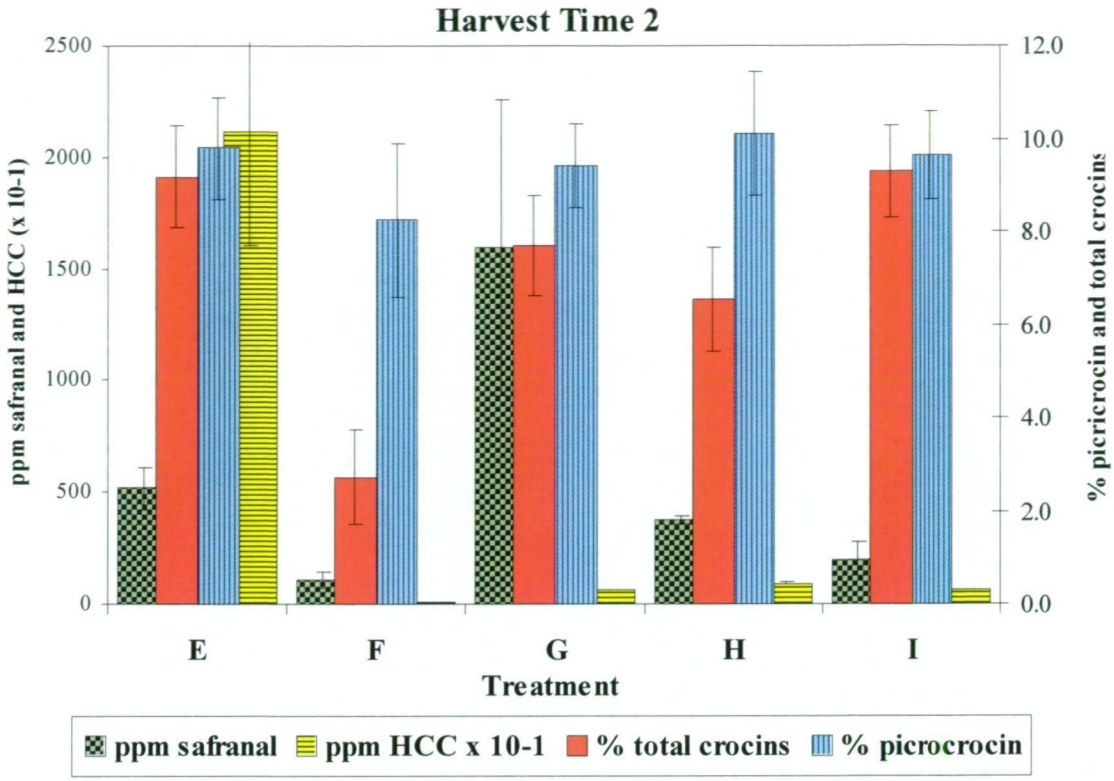


Figure 3-9: Drying treatments at harvest time 2 (see table 3-5 for treatment parameters). NB. While safranal and HCC contents are both given as ppm in this graph, the concentration of HCC plotted is reduced by a factor of 10 such that the highest level of HCC in this plot is approximately 10 X that of the highest level of safranal.

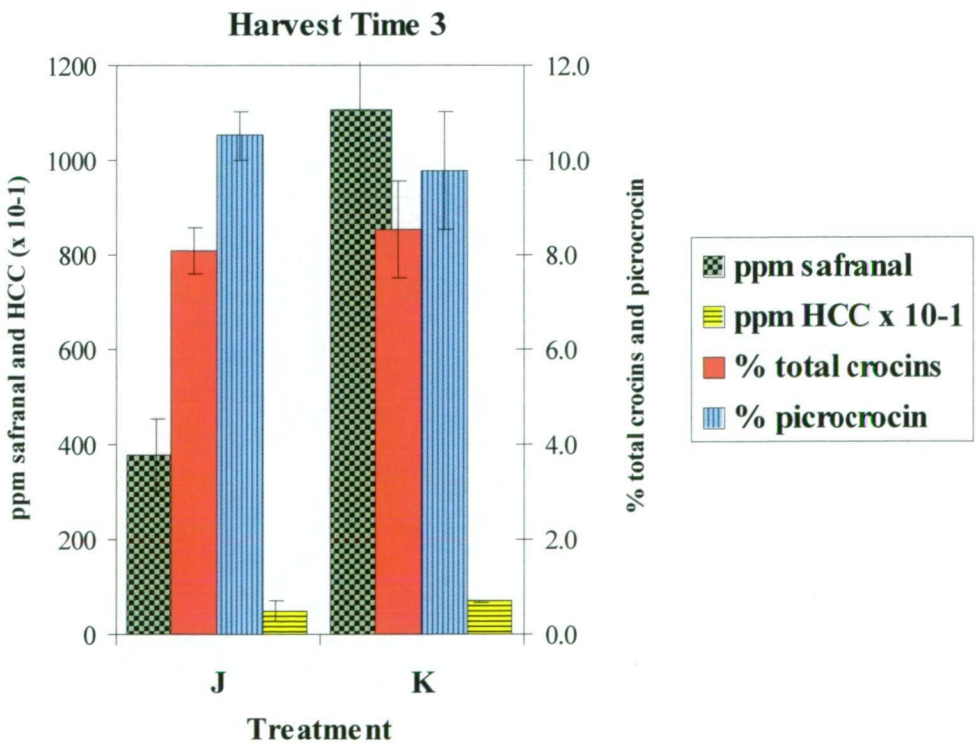


Figure 3-10: Drying treatments at harvest time 3 (see table 3-5 for treatment parameters).

3.C Testing of isolated drying parameters

3.C.1 Purpose Built Drying Apparatus

Because of the problems with temperature control and comparison of conditions between different drying apparatus highlighted from the preliminary experiments, it was decided to design and build a dryer specific to the requirements of drying saffron. This entailed a need for rapid heating from ambient to approx 100 °C, elimination of thermal inertia (resulting from a relatively large structural mass heated with a relatively powerful element and with crude thermostat control), and a requirement for variable fan speed control. There was also a consideration to keep the cost of the device relatively cheap, as the intention was that it would also be tested as a prototype dryer for commercial scale drying.

An aluminium cylindrical structure with a lid incorporating a mesh covered exhaust vent and 3 removable enamel coated aluminium mesh trays was constructed. Into this were installed 4 horizontally placed light fittings for light globes. These 200-watt bulbs were given 2 coats of "Pot Black" stove paint to block visible light that would cause photo-degradation of crocins. Between the lowest and next tray a thermocouple was installed for connection to the temperature controller. The controller connected to the bulbs was a Proportional Integral Derivative (PID) unit used to eliminate temperature overshoot and steady state error[*177*]. Directly above each tray, fittings were also placed for horizontal placement of glass thermometers to measure the temperature at each drying level. A variable speed fan was installed into the bottom of the apparatus directly above an intake vent.

This apparatus (which is illustrated in **figure 3-11**) was tested at a range of temperatures and fan speeds to determine the accuracy of temperature settings, heating times and variations in the temperature at different tray levels or in different positions on each tray. The following temperature variations were found where the temperature in the middle of the lowest of the three trays (where the thermocouple sensor was located) was always measured as that set ±1 °C. Without airflow the measured temperatures of the 2nd and 3rd trays up were respectively 1 °C and 3 °C below that set with the unit at 45 °C, and were respectively 5 °C and 15 °C below with the unit set at 100 °C. At all intermediate temperatures these differences where proportional for the temperature set over this range. With airflow set at a maximum 2.9 m/s as measured at the exhaust vent, these differences were decreased to <1 °C and 1 °C at 45 °C and to 2 °C and 6 °C when set at 100 °C. At intermediate airflows these measured variations where again proportional over the range.

The times taken for the unit to reach the set temperature were measured. For this and the later experiments the apparatus was always heated to the set temperature before the lid was removed and tray containing the samples added (to minimize heating time). Without airflow, the time taken to reach the set temperature after this was 40 sec. when set at 45 °C, and 3.5 min. when set at 100 °C. With full airflow these times were increased to 70 sec. and 8.5 min respectively with times proportional over the range for intermediate settings.

For all the following experiments the stigma samples were placed on the lowest tray of the three having in each case a temperature equal to that set and the timing of the drying duration at given temperatures was always taken from when the thermometer at this tray level registered the temperature set. Trays were always removed from the apparatus immediately the drying time had elapsed.

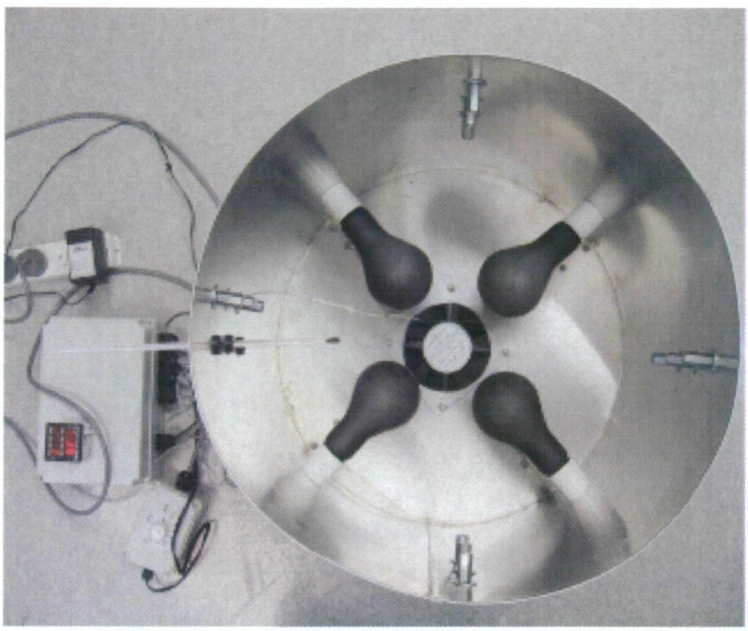


Figure 3-11: Overhead view of experimental saffron dryer (sample trays and lid not shown)

3.C.2 Temperature

Experimental

Flowers from a single day's harvest were transported and stored intact in sealed polythene bags at 4 °C until application of treatments in the laboratory. Treatments were conducted initially at 45, 55, 65, 75, 83, 90 and 97 °C with further periods, with the controller set at the lowest temperature of 45 °C following immediately to dry the stigmas to below the required 12% moisture (see table 3-5). The combinations of drying times at each temperature are detailed in **Table 3-6**. No airflow was applied to any of the treatments. For each treatment there were 4 replicate samples of approximately 25 stigmas and these were dried so that 2 replicates of each were dried in the treatment order 45-97 °C and the other 2 were dried in the order 97-45 °C so that each treatment had the same storage time.

After drying, each replicate was split into 3 sub-samples, 1 each for methanol and hexane extractions; and the remainder used for dry weight determinations. Extractions and HPLC and GC analyses were carried as before (see General Methodology 2A).

Table 3-6: Drying temperature treatments

Treatment	Duration at maximum temperature	Duration of subsequent drying at 45 °C	Final Moisture Content
45 °C	25 min.	60 min.	12.0%
55 °C	25 min.	50 min.	11.1%
65 °C	25 min.	40 min.	8.3%
75 °C	25 min.	25 min.	7.5%
83 °C	25 min.	10 min.	7.1%
90 °C	25 min.	0 min.	8.1%
97 °C	25 min.	0 min.	7.5%

Results and discussion

The results (**figure 3-12**) show that the greatest generation of safranal had occurred at elevated temperatures of 83 °C and above, with the highest content in the stigmas dried at 90 °C, though this was not significantly different (at the 0.05% level) to the 97 °C treatment. Both these means were significantly higher than all other treatments. The 90 °C treatment represented an 11-fold increase in safranal over the 45 °C treatment and 4.5 X that of the highest content of those treatments below 83 °C. Thus at some point above 75 °C, it appears that the direct thermal conversion pathway of picrocrocin to safranal becomes activated.

It is important to note that even in the treatments producing the highest safranal (dried at 90-97 °C) the saffron product still contained a picrocrocin content equivalent to a 22 fold molar excess of precursor and thus a great potential for further yield gains must exist.

The 65 °C and 75 °C treatments resulted in small but significant increases in safranal over those dried only at 45 °C, a result consistent with increased enzymatic activity driving greater conversion via the pathway involving the intermediate HCC (**see figure 1-2**). The significantly elevated HCC levels in the 45 °C and 55 °C treatments are consistent with conversion via this pathway, while the lower HCC levels at 65 °C and 75 °C probably reflect the increased conversion of HCC to safranal as dehydration was promoted with increasing temperature.

The results in **figure 3-12** also show that total crocin retention in the saffron was significantly enhanced at temperatures at or above 83 °C confirming the finding of the preliminary experiments. The lowest crocins level was measured in the 55 °C treatment, it being significantly lower than for all treatments above 75 °C, which is consistent with the theory that intermediate temperatures such as this allow maximum activity of enzymes responsible for colour degradation that become increasingly inactivated and then denatured as temperatures rise above this level. No attempt was made to apply treatments above 100 °C as such temperatures are known to cause significant destruction of crocin pigments through thermal degradation[*32, 35, 37*].

No significant differences were observed for picrocrocin levels amongst these treatments and this highlights the very large pool of the safranal precursor that remains in the stigmas after drying (approximately 22x the molar equivalent of the highest level of safranal).

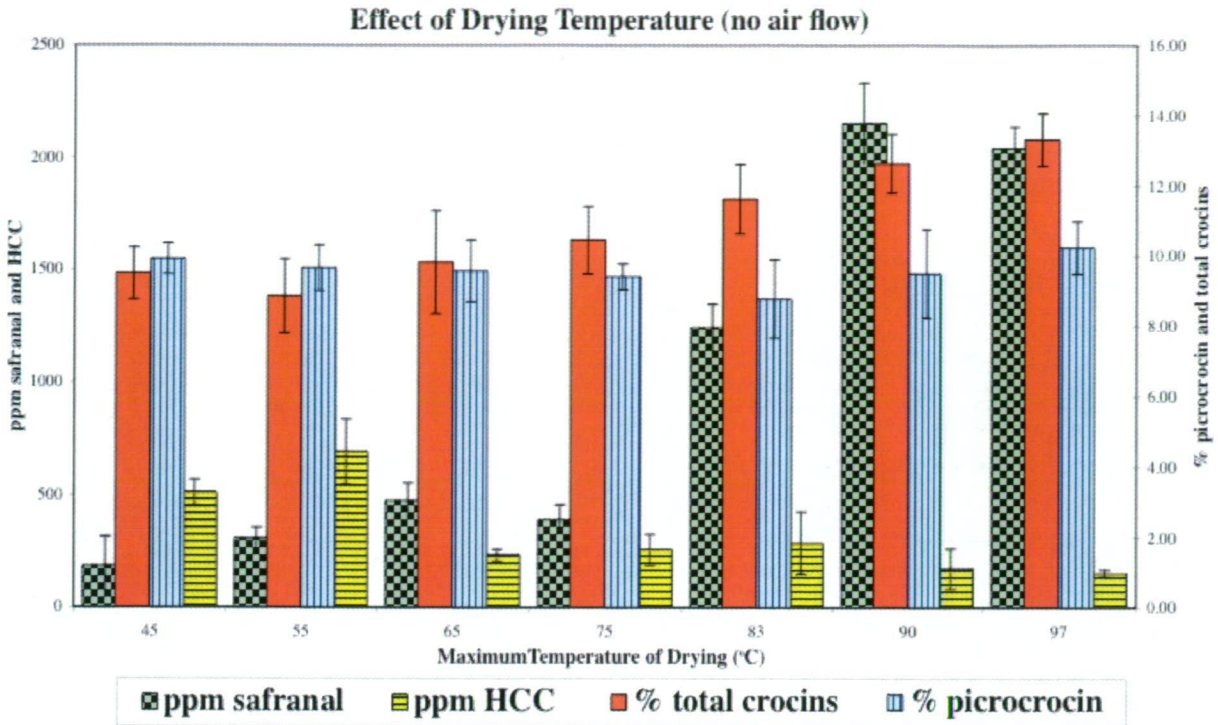


Figure 3-12: Effect of temperature on composition of saffron

3.C.3 Drying duration

Experimental

Stigmas, collected from a single days harvest and stored as previously described, were dried at 85 °C for periods of 5, 15, 25 and 45 min. with first 2 of these dried at 45 °C for a further 30 and 15 min. respectively to reduce the moisture to the desired level. Extractions, dry weight determinations and HPLC and GC analyses were carried out as previously described (see previous sub-chapters and General Methodology 1.A).

Results and discussion

The moisture contents of the saffron from these treatments were 17.9%, 15.6%, 12.2% and 9.52% respectively. The results (**figure 3-13**) shows that a drying period of 25 min. at 85 °C produced saffron with the highest safranal content, it being significantly higher than the 15 min. treatment, which was in turn significantly higher than both 5 min. and 45 min. The lower safranal in the 45 min. treatment must have been due to loss of the compound either through evaporation or thermal degradation to form unknown by-products, possibly isophorone related[*9, 30, 31*]. It has been proposed that intermediate water activities may favour the conversion of HCC to safranal in the enzymatic pathway (see **figure 1-2**)[*9, 35*] and this may also be true for the direct conversion as heating of preciously dried saffron has been shown not to result in increased safranal content despite a loss of picrocrocin[28, 29]. The picrocrocin content of the saffron in this experiment was also significantly reduced after 45 min. suggesting that compounds other than safranal had been formed from it, though whether via safranal is not known.

These results may therefore indicate the length of period required to pass completely through the favourable water activity range at this temperature, specifically > 15 min and probably well short of 45 min. (i.e. approximately 25 min.).

Figure 3-13 also shows a gradual degradation (thermal) of total crocins with increasing drying time at 85 °C and though this loss is not significant between 15 and 20 min., the content in the 45 min. treatment is significantly lower than for all other periods.

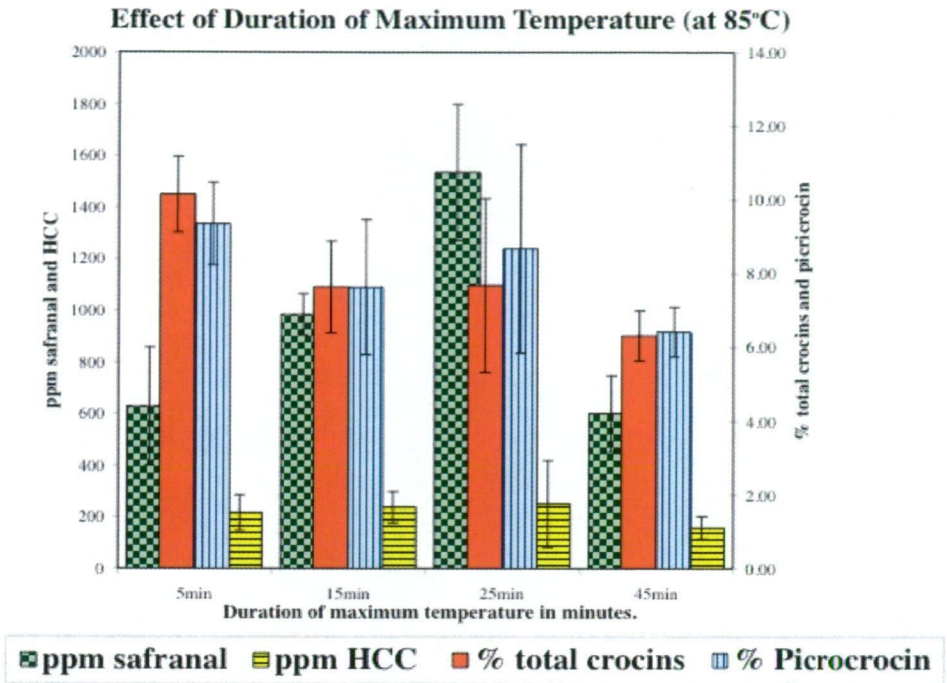


Figure 3-13: Effect of duration of elevated temperature on components of saffron

3.C.4 Airflow

Experimental

Flowers from a single harvest were transported and stored intact in sealed polythene bags at 4 °C until application of treatments in the laboratory. Airflow treatments were performed at 2 temperatures; 45 °C to approximate the current Industry drying temperature, and at 83 °C representing the conditions where much greater safranal is produced by direct thermal dehydration. Two 45 °C treatments were applied with the fan on full (2.9 m/s) and with it off, for drying periods of 60 and 90 min. respectively. The 83 °C treatments involved a range of exhaust vent airflows, specifically 0, 0.5, 1.5 and 2.9 m/s. These were all dried for 20 min. at 83 °C before being turned down to 45 °C and dried for a further 30 min. each.

To gain an estimation of the comparative safranal loss from evaporation during these treatments, a solid phase micro-extraction device (SPME) was employed (Supelco 100 μm polydimethylsiloxane coating). This devise was placed vertically in the grate of the exhaust vent with the sampling needle extending into the drying chamber exactly 1 cm for the period

of each drying treatment. After each run the needle was desorbed into the injecting chamber of the GC using the same column and temperature profile as for all other saffron extracts. No attempt was made to calibrate the actual levels of safranal by this method, as the relative response of the GC detector should give a proportional measure of safranal being lost through the vent, assuming non-saturation of the SPME needle coating. The needle was conditioned at 290 °C in the GC between sampling runs.

Extractions, dry weight determinations and HPLC and GC analyses were carried out as previously described (see previous sub-chapters and General Methodology).

Results and discussion

At 45 °C the moisture content of the saffron produced was 13.1% with airflow and 14.2% without. At 83 °C, the moisture contents of the saffron produced were 13.89%, 12.63%, 11.58% and 11.02% for the airspeeds 0 - 2.9 m/s respectively.

The results of the analysis of saffron these 2 experiments are shown in **figures 3-(14&15)**. The use of airflow to assist in drying stigmas had clearly had a detrimental effect on the safranal level at both temperatures with significant differences between all treatments in both experiments. These results support the proposal (stemming from the preliminary experiments) that airflow is detrimental to safranal production and/or retention. This effect was slightly greater over the airflow range used at 83 °C but still considerable at 45 °C. Certainly the continual removal of the headspace, particularly at elevated temperature would cause a loss of safranal even though it has a boiling point (217 °C) well above this temperature.

This conclusion is confirmed by the relative measures of safranal from GC analysis of the SPME sampling. This gave mean areas of 200 ± 13 and 1688 ± 108 for the 45 °C treatment with and without airflow respectively, and mean areas of 254 ± 21 and 1731 ± 46 at 83 °C with and without airflow respectively. While the responses for safranal loss are only comparable within each temperature treatment due to the very different adsorption characteristics of SPME coatings at different temperatures, it clearly shows that significant loss of safranal occurs due to greater exchange of airspace with the use of the fan.

The faster rate of drying caused by airflow may also play a role in limiting safranal production in that it reduces the time where the stigmas are in the favourable water activity range for the dehydration steps of both pathways to occur. This is supported by the significant accumulation of the intermediate HCC in the 2.9 m/s treatment at 45 °C (**figure 3-14**) indicating that while enzymatic production of HCC had been favoured, the 2nd dehydration step had been negated at some point.

The rate of drying may also play a role through differences in the cellular structure or porosity of the saffron produced[*32*] and thus on the availability of the crocins for solvent extraction . This appears to be the case with the effect on pigments as the saffron dried without airflow exhibited significantly higher extracted crocins content than all other treatments at this temperature. No such difference was apparent at 45 °C where enzymatic degradation occurring in these conditions may have masked any such effect.

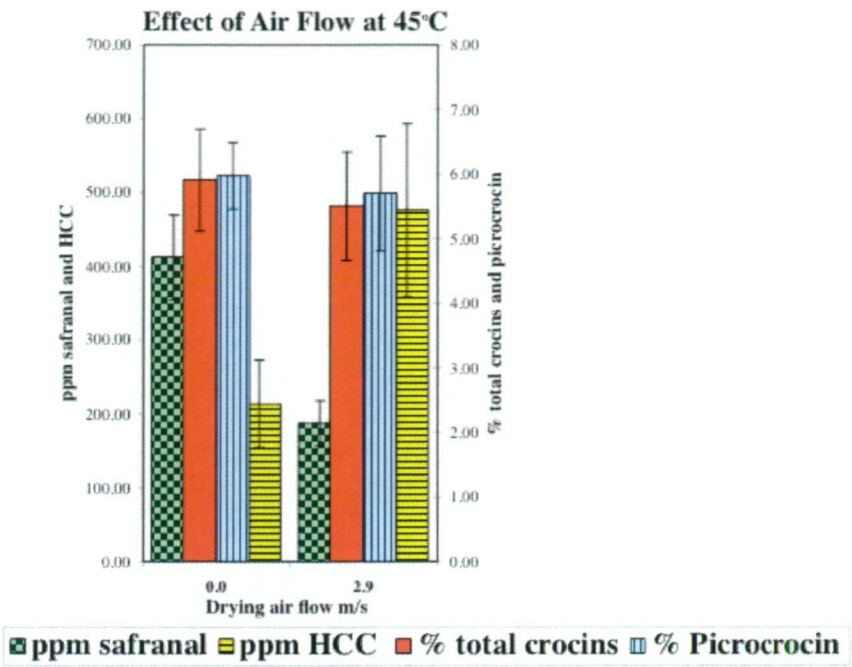


Figure 3-14: Effect of airflow at moderate temperature on components of saffron

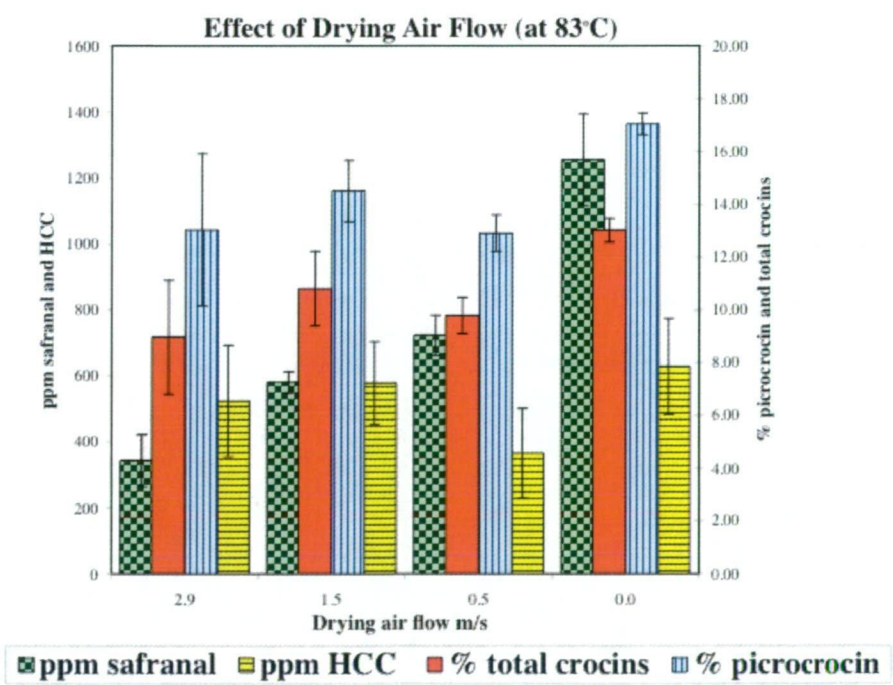


Figure 3-15: Effect of airflow at elevated temperature on components of saffron

3.C.5 Implications

For the experimental results described above, each treatment factor being tested was isolated from other factors by using only stigmas harvested on the same day and dried in the same device. These results clearly show that saffron quality can be enhanced by drying at approximately 90 °C without airflow for approximately 25 min (although this time may be specific only to the specific drying device used). This improvement is principally through increased safranal production and/or retention, but also involves greater retention and/or availability of pigments, though the reasons for this are not clear. Moreover, given that only a small proportion of the picrocrocin was converted to safranal under these conditions, there is still a great potential for further aroma generation and thus elucidation of the process of safranal formation remains incomplete.

The nature of the drying process (especially the degree of enclosure of the drying device and the use of airflow), together with the mass of fresh stigmas being dried, will combine to determine the relative humidity of the air surrounding the stigmas. Thus different drying methods should result in a variation of humidity surrounding stigmas leading to differing drying rates. Given this and the report that the release of volatile aglycones such as the generation of safranal from hydrolysis of picrocrocin is favoured by intermediate water activity[*35*], the need to investigate the role of humidity in determining the degree of aroma generation becomes clear. Humidity may also impact upon colour retention and/or colour availability if different drying conditions do in fact result in differences in filament structure from which pigments are more or less readily dissolved.

3.D Measurement of "actual" stigma temperature and the role of humidity during drying

As Carmona and Alonso[*28*] have discussed, the understanding of exactly how aroma (safranal) is generated during a variety of saffron drying processes is not as clear as has previously been believed. Central to this problem is the large excess (>20 times molar equivalent of safranal) of picrocrocin remaining in saffron[*34, 85*] and why greater conversion to safranal cannot be generated *in vivo* either from initial drying or from further high temperature treatment (at ≈ 90 °C) after initial drying[*32*].

It has been reported that the release of volatile aglycones such as the generation of safranal from hydrolysis of picrocrocin is favoured by intermediate water activity (a_w ~ 0.43-0.53)[*35*]. The following experiments were therefore designed to test the hypothesis that there is a critical range of water activity through which the stigmas pass during the drying process and that by slowing this process through application of increased humidity at a favourable temperature the stigmas remain in this range for longer and more hydrolysis may take place.

3.D.1 Application of elevated humidity drying treatments

Introduction

The effect of humidity during the drying process was examined by comparison of drying treatments involving varied levels of humidity in the drying chamber with both the current commercial method and the high temperature optimum (without added humidity) determined from Chapter 3.C. The effect of drying duration at elevated temperature was also tested.

Experimental

Flowers from a single days harvest were transported and stored intact in sealed polythene bags at 4 °C until stigma separation and application of treatments in the laboratory later that same day. Stigma drying treatments (with 4 replicates per treatment) were divided into two separate experiments.

The application of elevated humidity for these experiments required a more enclosed drying space than the experimental dryer could provide and so an oven was used once again. In this case a 25-litre *Thermoline* gravity convection oven with a PID temperature controller was selected as this provided the most enclosed a chamber available (without being pressurised) with as precise temperature control as possible.

The first experiment involved application of elevated humidity drying treatments (at 90 °C) in comparison to the current commercial method (food dryer) and elevated temperature (at 90 °C) treatments using both the oven and the experimental dryer (used in 3.C). Three different levels of relative humidity (RH) were achieved by placing a bowl containing either distilled

hot water or saturated solutions of salts (Potassium sulphate or Sodium chloride) into the bottom level of the oven prior to introduction of the stigmas for drying. The solutions were made up by boiling water that was immediately poured into the bowl containing the salts and introduced into the oven. The oven was then allowed to equilibrate back to 90 °C prior to introduction of the stigmas samples.

Relative humidity was measured and logged in each drying device using a Barnstead/ERTCO HiTemp102RH Recorder that was placed immediately adjacent to the stigmas (and set at a recording interval of 10 seconds). Prior to the drying experiments, this device was calibrated against a series of saturated salt solutions placed in the chamber where the %RH was also calculated using the wet and dry bulb thermometer technique[*72*]. The conditions involved in each of the drying treatments including the humidity range (from time of stigma introduction to the end of the drying period) are detailed in **Table 3-6**. For the treatments in the more enclosed space of the oven, the final RH value represents an approximate equilibrated humidity (at least while liquid remained in the bowl to maintain that humidity level).

The second experiment involved application of the highest humidity treatment at 90 °C over a range of drying times to determine the optimum duration at this RH for safranal production and pigment retention.

The saffron samples produced from treatments in both these experiments were analysed as for previous drying experiments as described in General Methodology (2A). The arrangement of the bowl and humidity recorder is illustrated in **Figure 3-18**.

Table 3-6: Treatment descriptions for elevated humidity drying experiment (see figure 3-16). RH % numbers given are the humidity range inside each device from immediately after introduction of stigmas to the final RH at the end of the drying run.

Treatment	Drying device	Conditions
A	Food dryer	45 °C with airflow for 90 min (RH 55→6%)
B	Experimental dryer	90 °C no airflow for 30 min (RH 78→3%)
C	Oven	90 °C no airflow for 30 min (RH 85→3%)
D	Oven	90 °C no airflow for 60 min (RH 98→31%)
E	Oven	90 °C no airflow for 60 min (RH 99→36%)
F	Oven	90 °C no airflow for 60 min (RH 100→45%)

Results and discussion

Figure 3-16 shows the effects of different levels of added humidity during drying on the contents of the main secondary metabolites in the resulting saffron. These were compared to saffron dried using the current industry method and also a treatment using the prototype dryer (as used for the 2005 experiments). This latter method produced saffron with a safranal level comparable to that dried in the oven without added humidity. All the treatments produced saffron with moisture contents below 12%.

The addition of added humidity had a significant promontory effect on safranal production and/or retention with the highest content of safranal resulting from the highest humidity treatment (RH = 42% with water) although the difference between this and the treatments

employing saturated solutions (32% and 36%) was not significant. This treatment produced saffron with a safranal level approximately 2.5 times that of saffron dried at 90 °C in either the oven or the prototype dryer without added humidity and over 6 times that dried by the current commercial method. The results therefore, support the hypothesis that by slowing the drying process at raised temperature, the stigmas remain in the critical range of water activity for longer, allowing more conversion of picrocrocin to safranal to occur.

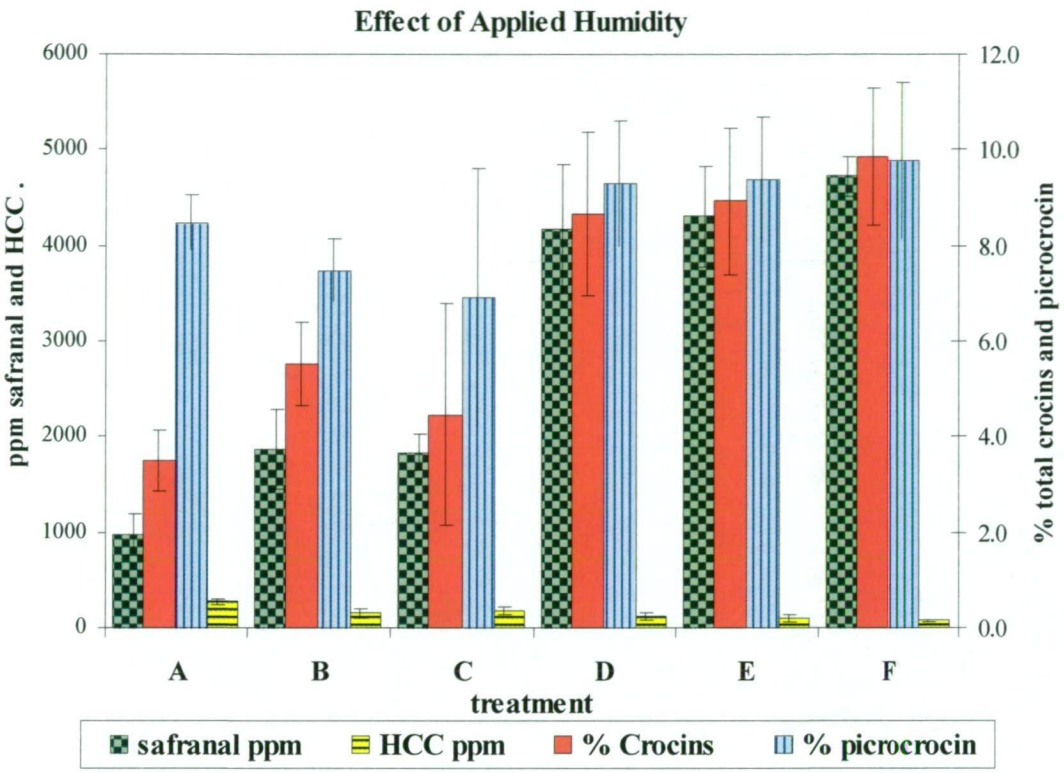


Figure 3-16: Compositional changes with different (applied) humidity treatments at ≈ 90 °C compared with current commercial drying method at 45 °C.

The 3 elevated humidity treatments (D, E & F) also resulted in significantly greater retention of crocin pigments (all $Pr > |t| < 0.02$). This implies either that processes such as enzymatic degradation (by polyphenoloxidases) responsible for crocins loss had been prevented or that the saffron produced had a greater availability of pigments. Given that the extraction time for the HPLC-UV determination of crocins was 16 hours, any pigments not extracted in that time might be considered "unavailable" for commercial usage anyway. Moreover, if greater pigment availability due to increased porosity of filaments is a result of more rapid drying (as proposed by Carmona *et al.*[*28, 32*]), then treatments B and C (90 °C without added humidity) would be expected to produce the most porous saffron. The possibility remains that by drying in elevated humidity at ≈ 90 °C, enzymes responsible for crocin degradation may be inactivated as stigmas heat more rapidly above a critical temperature (of inactivation) due to reduced evaporative cooling.

The interpretation of the picrocrocin contents is less clear as significant differences were not observed between many of the treatments. This may have been because of the relatively large error for some of these treatments. The only significant difference found was lower picrocrocin level in treatment B compared to the elevated humidity treatments (D, E & F). This suggests that at 90 °C without added humidity picrocrocin is lost, possibly due to β-

glucosidase activity, but without safranal being formed. It may be postulated that at high temperature, HCC (the product of this activity) is degraded to form compounds other than safranal. This supports the findings in chapter 3.B (**figure 3.9**) where very high HCC levels (in frozen/thawed stigmas) disappeared upon drying at elevated temperature, but without being converted into safranal.

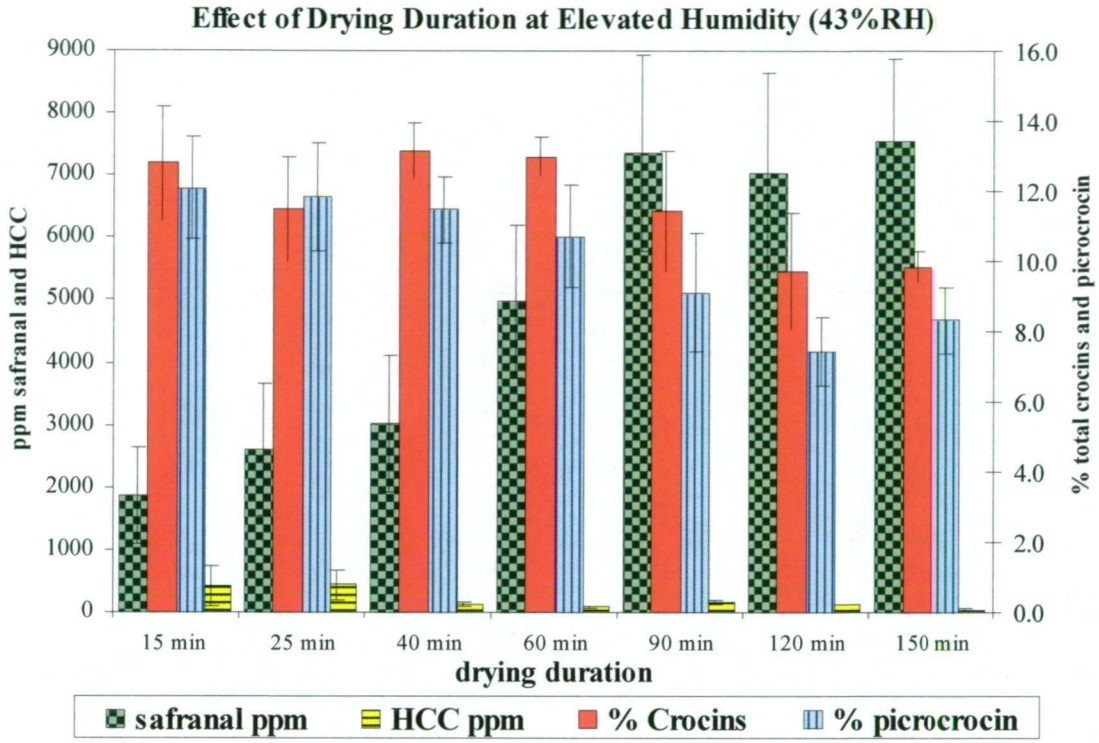


Figure 3-17: Effect of drying duration at elevated temperature (90 °C) and humidity on composition of saffron.

The comparison of drying duration times using the highest humidity treatment (equivalent to F in **figure 3-16**) is illustrated in **Figure 3-17**. It should be noted that drying times < 60 min. produced saffron that was not sufficiently dry (i.e. > 12% moisture). Above 60 min. duration, significantly more safranal was produced (all $Pr < 0.03$) than for shorter drying times, but no significant gain was detected beyond 90 min.

It is also apparent that after 150 min. a net loss of crocins occurred. This may be due to thermal degradation once the stigmas had dried out, a form of pigment loss reported by others[*9, 28, 35, 37*]. By the latter times (120-150 min.) there was also a significant loss of picrocrocin. This loss (approximately 40,000 ppm) is partly attributed to the 7000 ppm safranal gain (a molar equivalent of approximately 12,000 ppm of picrocrocin). Evaporation of safranal and/or conversion of picrocrocin to compounds other than safranal may account for the discrepancy.

Implications

It is clear that the application of elevated humidity at high temperature greatly enhances safranal generation in saffron and may also prevent pigment degradation. The optimum duration of drying (in the device used) was between 60 and 90 min. To more clearly explain

the temperature profiles during drying, it is necessary to actually measure the temperature of stigmas during such treatments to determine the effects evaporative cooling.

3.D.2 Measurement of humidity and "actual" stigma temperatures during drying

Introduction

The hypothesis that evaporative cooling during drying must lower the temperature of stigmas (and thus is a significant factor in determination of optimal conditions for safranal production) was tested. This was achieved by measuring the actual temperature of stigmas during drying runs in the oven and food dryer, with and without added humidity. This was plotted against the temperature and relative humidity (RH) of the oven/dryer space.

Experimental

Data was collected from drying treatments including those performed in Chapter 3.D.1 as well as further drying runs performed both in the laboratory and on farms as part of the commercial scale application of the new drying conditions (see Chapter 3.F). In each case the stigmas used were harvested on that day. The 2 drying treatment used were in the oven, either at ambient RH (equating to treatment C in Chapter 3.D.1) or with distilled hot water in the bowl (equating to treatment F in Chapter 3.D.1). The treatments were replicated on 6 days for the dryer and oven at ambient humidity and on 10 days for elevated humidity.

The temperature and relative humidity (RH) inside the oven/dryer were measured and logged using a Barnstead/ERTCO HiTemp102RH Recorder that was placed immediately adjacent to the stigmas during the drying experiments (as for 3.D.1). The actual temperature of the stigma tissue was also measured by wrapping a bundle of 10 stigmas around the sensor of a K type thermocouple with fine cotton thread (see **figure 3-19**). The arrangement was made such that the head of the thermocouple was positioned at the point where the stigmas began to spread away from the fine cotton teflon thread used to tie the bundle so that contact with the stigma tissue was maintained without being too tightly suppressed and hinder water loss. It should be stressed that this was only an approximation of the density of stigma tissue as it would normally be when dried inside the oven or dryer and involved a degree of subjective judgement about this positioning. This arrangement was inserted into the dryer via the central exhaust vent or into the oven chamber via a lateral sensor port and attached to a digital temperature meter and is illustrated in **figure 3-18**.

The logged data from the HiTemp/RH recorder were synchronised with the stigma temperature recordings form the digital meter (recorded by hand) and the means and standard deviations for each of these measurements calculated using *Microsoft Excel 2003l.*

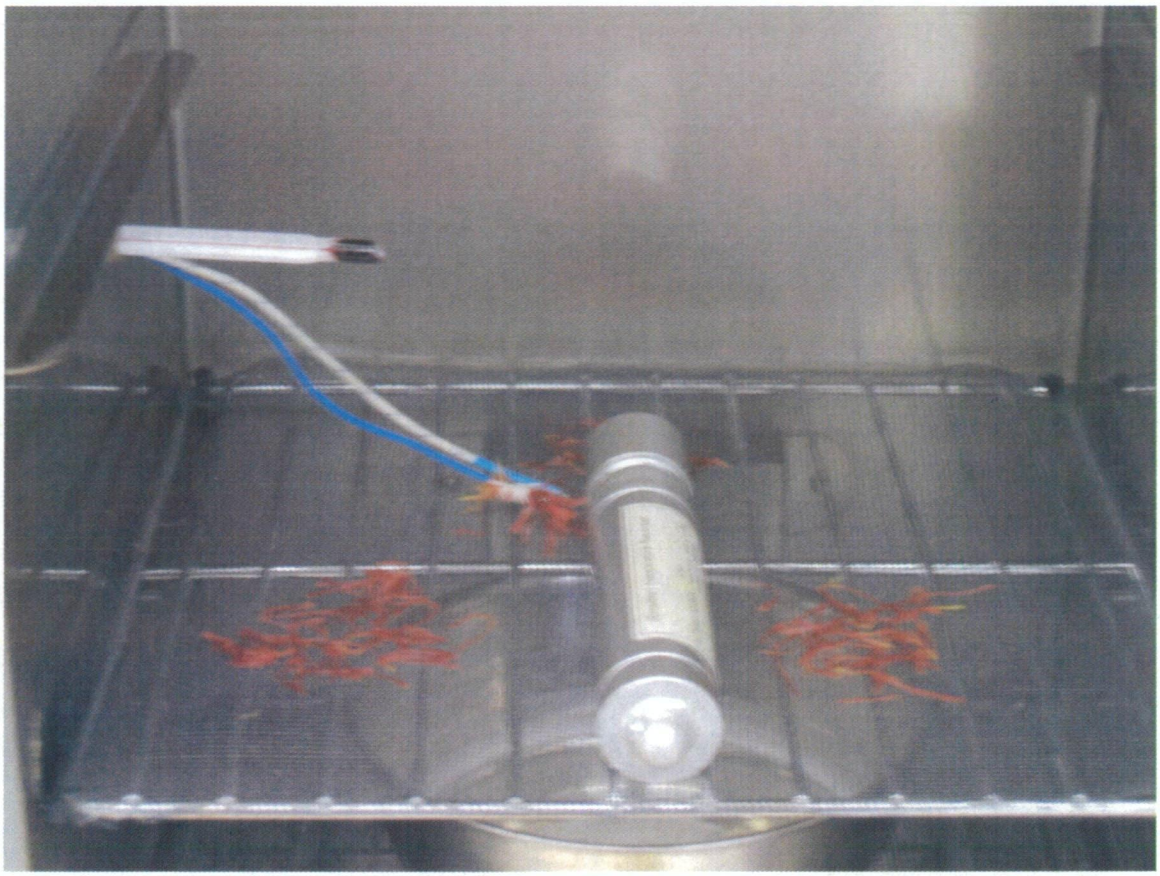


Figure 3-18: Interior of oven showing stigma bundle attached to thermocouple, stigma drying replicates, humidity/temperature logger and water/solution dish placed beneath

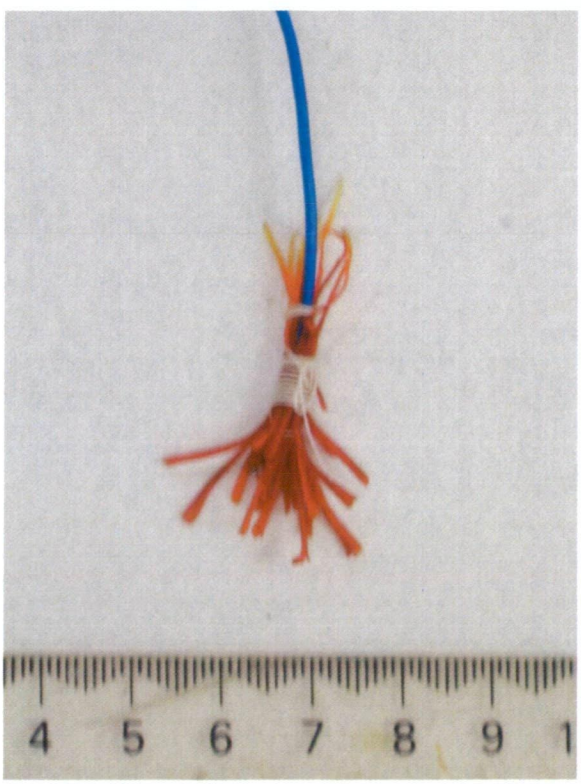


Figure 3-19: Arrange of stigmas tied in a bundle around thermocouple wire placed where the stigmas begin to spread away from the thread to prevent excessive compaction.

Results and discussion

The plots of air temperature, % RH and stigma temperature for drying runs at low temperature (food dryer) and at high temperature without (i.e. at ambient RH) and with added humidity (hot water), are illustrated in **figures 3-(20-22)**. In the food dryer set at 45 °C the internal humidity dropped from ≈ 50% to < 15% relatively rapidly (20 min.) due to airflow removing the headspace around the stigmas. A small degree of temperature suppression is evident in **figure 3-20**, peaking at ≈ 10 °C after 20 –30 min. and the stigma temperature did not reach that set (on the dryer thermostat) until > 100 min.

Both high temperature (oven) plots show that the RH was high (>80%) immediately after sample introduction but then equilibrated over the runs. The oven chamber was not perfectly sealed and thus humidity dropped as excess moisture was lost. For the run without added hot water the initial humidity was due only to the moisture in the stigmas introduced to the hot oven at time 0 and the humidity then equilibrated to 5% after 20 min. For the runs with the hot water added the initial 100% humidity would have been due to both the moisture in the stigmas and (more significantly) the water vapour from the hot water added at the same time. The humidity in this run then equilibrated to 42% over 50 min. The equilibration humidity of the oven, when hot saturated solutions of sodium chloride or potassium sulphate where used instead of water, was measured at 31% and 36% RH respectively.

It should be noted that the temperature recovery time (to 90 °C) of the air space inside the ovens was much longer when the moisture was introduced via fresh stigma samples (30 min) or stigmas plus added humidity (> 40 min) compared to when only the recorder was introduced (5.5min).

A cooling effect due to evaporation of moisture content of the stigmas was apparent resulting in the suppressed temperature of those stigmas. Without added humidity (**figure 3-21**), this effect was marked as the stigma temperature remained below 70 °C until the stigmas dried out. This occurred after 23 min. With added humidity (**figure 3-22**) the stigma temperature initially rose to a higher level (approximately 65 °C compared to 50 °C) after 10 min due to the higher humidity of the chamber, but then rose more gradually to 90 °C as the stigmas took much longer (90 min.) to dry out. From this it may be inferred that the (optimum) temperature at which safranal generation occurs is actually below 90 °C (i.e. in the range 65-90 °C) and suggests that previous saffron drying studies[*32, 38, 45, 68, 69*] contain inherent errors, where reported temperatures of ovens or other drying devices were assumed to be critical instead of the actual stigma temperature.

With added humidity the stigmas more rapidly heated to a temperature where thermal hydrolysis of picrocrocin to form safranal may begin to occur. Then, because it takes much longer for them to dry out, they remained within an intermediate water activity range for much longer (probably > 60 min.), allowing much greater safranal generation. Such a mechanism would explain why the previous highest safranal levels have been reported from Spain[*33*] where relatively large masses of stigmas are subjected to heat sources ≈ 100 °C without being greatly spread out [*10, 32, 33, 69*]. Such drying would maintain relatively high humidity inside the mass such that the stigmas in the middle would require a significant period of time to dry out.

The 90 min. required for the stigmas to dry out with added humidity supports the conclusion from the previous chapter (see **figure 3-17**) that approximately 90 min is the optimum time of drying for this method to achieve full development of safranal content with minimal pigment loss. It should be noted that this time would only apply to this configuration of oven type and size, water volume and stigma mass.

The temperature lag (below 60 °C) exhibited in the stigmas in **figure 3-21** supports the proposition that enzymatic degradation of crocins may occur for longer in the oven without added humidity (as proposed in the previous chapter 3.D) and that the "blanching" effect (alluded to in chapter 3-B) is more immediate in the humid conditions.

The occurrence of the temperature lag does also raise the possibility that some of the safranal production may be attributable to enzymatic conversion depending on the inactivation temperature of the specific β-glucosidase in *Crocus sativus;* something not precisely known.

Implications

These findings further support and explain the proposition that high temperature/humidity drying produces more safranal, and acts to preserve the pigment contents of the saffron. The increase in safranal production may be due to humidity reducing the degree of temperature suppression while increasing the drying time. The possibility still remains however, that the differences in crocin contents observed may be in part due to different porosities affecting pigment availability rather than due to a "blanching" effect.

To this point the application of the high temperature/humidity method had only been conducted on a laboratory scale comprising < 100 stigmas split into 4 replicates per treatment. To assess viability of transfer of the method to commercial drying, a "scaling up" of the method during a normal harvest is required.

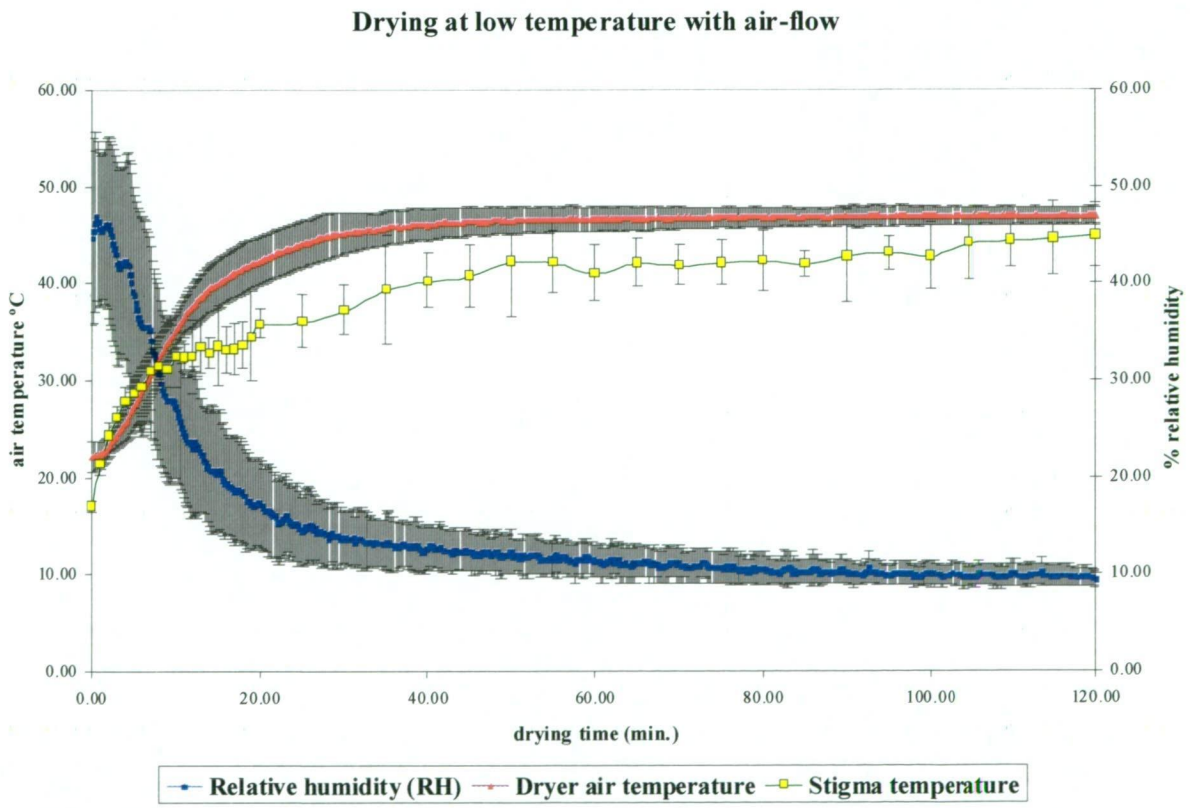


Figure 3-20: Plot of temperatures and humidity during low temperature drying (in the food dryer) using airflow. Error bars represent standard deviations at each recording time (and appear as solid black either side of the mean values for the dryer humidity and temperature which were recorded every 10 sec.).

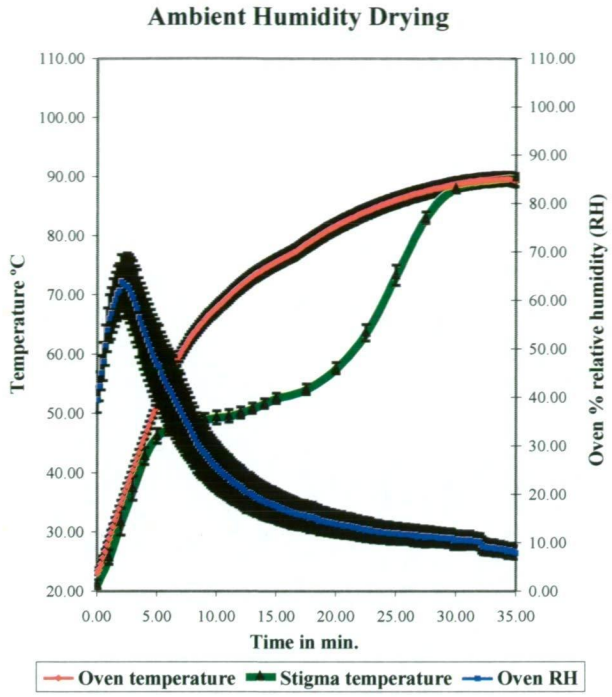


Figure 3-21: Plot of temperatures and humidity during high temperature oven drying without added humidity. Error bars represent standard deviations at each recording time (and appear as solid black either side of the mean values for the oven humidity and temperature which were recorded every 10 sec.).

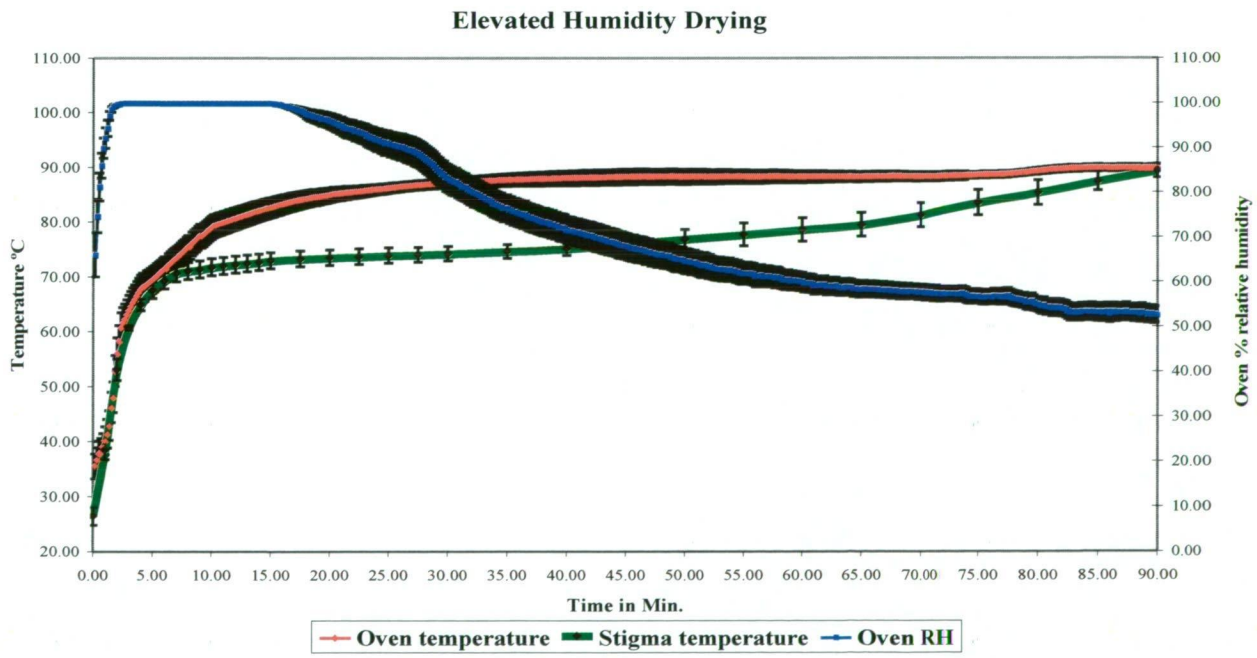


Figure 3-22: Plot of temperatures and humidity during oven drying at elevated temperature and humidity. Error bars represent standard deviations at each recording time (and appear as solid black either side of the mean values for the oven humidity and temperature which were recorded every 10 sec.).

3.E Optimal timing of flower harvest

3.E.1 Degree of flower opening

Introduction

It is widely reported in the literature that saffron flowers should be picked early in the day to minimise exposure to sun and wind that may cause premature drying and photo-degradation of pigments[*2, 10, 61*]. This recommendation may have simply arisen from practical commercial experience, as there are no reports of experimentally tested evidence to support it.

Critical to the matter of harvest timing would seem to be the degree of flower opening and thus exposure of the stigma to the elements. The tendency is for the majority of flowers to emerge overnight before the tepals gradually open during the day, although some flowers do emerge later in the day. The question is therefore posed as to whether it is preferable just to pick flowers early in the day (leaving flowers that emerge later in the day for the next mornings harvest), or rather to pick as many flowers as possible with the tepals closed whenever they emerge.

The purpose of the following experiment was to test the hypothesis that the degree of flower opening is an important factor determining the quality of the saffron that results from subsequent drying; irrespective of when during the day flowers emerge.

Experimental

Flowers were harvested from a single farm at 2 times on the same the day. The first pick was at 8 am; and any flower buds that had not fully emerged were left (as per usual commercial practise) for a later pick at 3 pm when elongation of the peduncles had brought these flowers sufficiently above ground to be picked. The weather conditions on the day were sunny with light winds and a maximum air temperature of 23 °C occurring at approximately 2 pm. The air temperature at 8 am was 9 °C while at 3 pm it was 22 °C. The flowers from both these picks were immediately sorted into 3 categories: closed, partially open and fully open. The partially open flowers were not used for this experiment to minimise the subjective error in judging degree of openness.

The flowers were transported to the laboratory in sealed containers at 4 °C, where stigmas were separated and each picking treatment was split into 4 replicates and dried at 90 °C in the oven at elevated humidity (hot water dish ≈ 100→45% RH as per Chapter 3.D). The resulting saffron was analysed as for previous saffron drying chapters as described in General Methodology (2.A).

Results and discussion

The effects of the flower picking treatments on subsequent saffron quality (components) are shown in **figure 3-23**. From this graph it is apparent that the saffron produced (by this drying method) from the stigmas of open flowers had significantly ($Pr < 0.0001$) reduced levels of crocin pigments and picrocrocin compared to that from closed flowers. This was irrespective of when during the day the flowers were picked although a smaller timing effect was also evident. From ANOVA comparison of the treatments, significant reductions ($Pr <$

0.0001) in the final content of picrocrocin resulted from flowers picked in the afternoon (comparing open with open and closed with closed). Similarly, a significant reduction (Pr = 0.0006) of total crocins resulted in saffron produced from open flowers picked in the afternoon although for closed flowers the difference was not significant (Pr = 0.093). It was noted that from a test for interactive effects between flower opening stage and picking time, no such effect was apparent for picrocrocin, but an effect approaching significance (Pr = 0.0729) was found for the pigments.

These differences may be interpreted as due to the exposure of stigmas to light and open air (with or without airflow (wind). Enzymatic degradation of picrocrocin to produce HCC may occur in the stigmas at increasing rate as the temperature warms during the day and this process may be driven further as flowers open and stigmas begin to dry, converting HCC to safranal. The loss of crocins in open flowers is attributable to photo-degradation and this effect would be accentuated through the middle of the day with the sun higher in the sky (giving the possible interactive effect described above).

It should be emphasised that the safranal contents of the saffron produced via this high temperature/humidity method would not reflect the contents in the stigmas prior to drying or if the saffron had been dried at a lower temperature. No significant differences in safranal content were found between treatments except between the early picked closed flowers and the late picked open flowers (Pr < 0.0001) that showed a small reduction. This may simply reflect the smaller pool of picrocrocin (available for conversion to safranal) in the stigmas from the latter picking treatment.

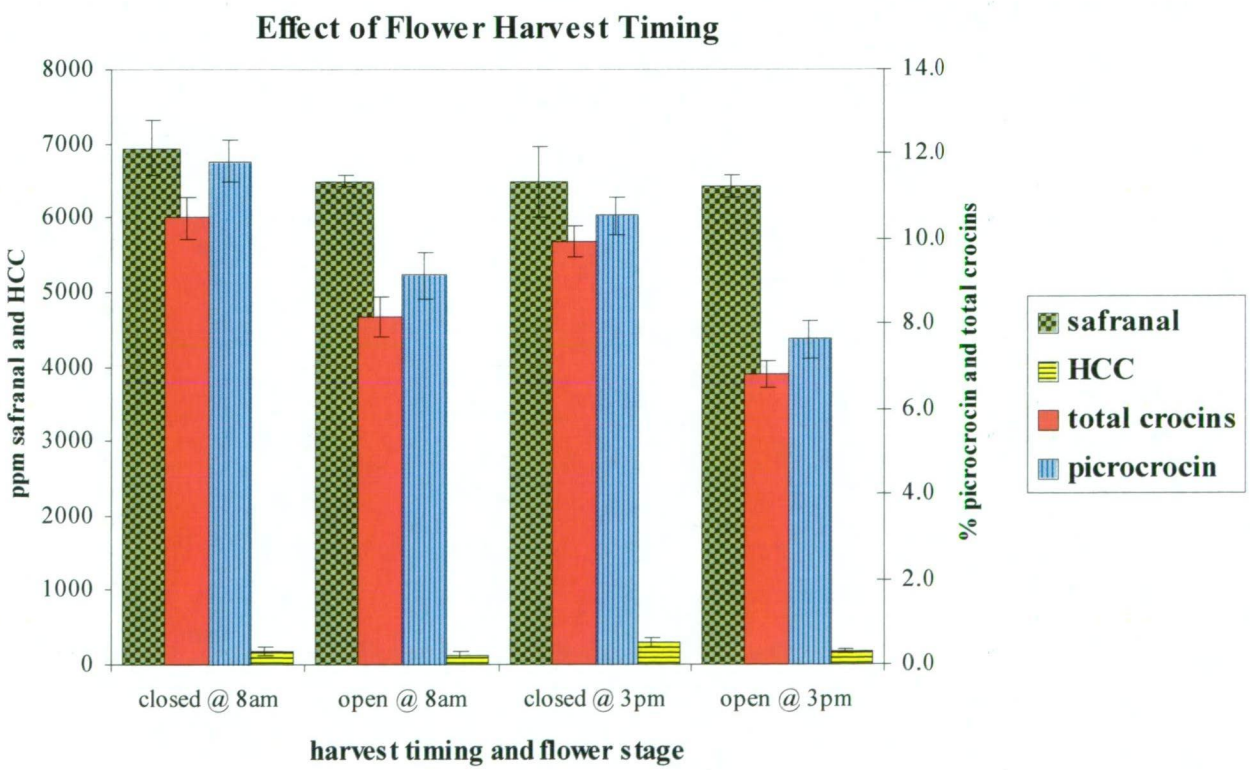


Figure 3-23: The effect of harvest timing and degree of flower opening on the quality of saffron from subsequent drying.

The only significant difference in HCC content was between closed flowers picked at 3 pm and all other treatments. Interpretation of this is problematic as the high temperature of drying might be expected cause degradation of HCC[*28, 68*], either into safranal or other products. It is possible however, that this difference reflects a greater rate of β-glucosidase activity in the stigmas of the closed flowers during the warmer middle part of the day, but without the drying effect of occurring in open flowers converting this to safranal. This would require that the high temperature/humidity conditions of this method do not result in complete degradation of any HCC generated prior to drying; and certainly some HCC was still detectable from the earlier testing of the method (see figures 3-(12&13) and 3-16).

Implications

It is clear from this work that it is preferable to pick flowers early in the day and just before they open (providing that the flowers have fully emerged) to minimize pigment loss. What is also apparent is that it is desirable to pick flowers during the day (and preferably before they open) rather than leaving flowers that emerge later in the day for then next mornings harvest (a common practise[*5, 10*]). Besides pigment loss, such practice will also prevent significant loss of picrocrocin allowing the full potential for safranal generation during high temperature/humidity drying to be realised.

3.F Commercial scale application of elevated temperature drying

Introduction

In the preceding chapters (3.B-3.D), the experimental determination of improved drying conditions (giving saffron with greater safranal and crocin pigments contents) was conducted on a relatively small scale in the laboratory. To test the practicality of applying these conditions to commercial scale drying these methods needed to be transferred farms where all the stigmas harvested during a day where to be dried. Such trials was conducted over 2 harvest seasons; the first testing the high temperature method and the second the high temperature/humidity conditions, with both compared to the current commercial method.

3.F.1 High temperature method

Experimental

The same (*Thermoline*) oven that was used for the laboratory experiments was transferred to 1 of 4 farms on each of 10 days for the drying trials. Half of the total crop of flowers harvested each day was dried in a food dryer according to the normal commercial method (≈ 45 °C for 60 min.) and the remaining half dried in the oven set at 90 °C for 35 min. Where the saffron from the oven treatments appeared to be clearly too dry for easy handling (i.e. brittle), the filaments were allowed to stand on oven racks at ambient temperature in a darkened room to re-adsorb moisture (equilibrate).

Sub-samples of approximately 500 mg of saffron from both these treatments were split into 2 replicates for later extraction and analysis (as for preceding laboratory experiments) as described in General Methodology (2.A).

Results and discussion

The total number of flowers picked on each day's harvest ranged between approximately 300 and 800 so that the number of stigmas for each replication of both drying methods was between 150 and 400. All of the saffron samples from both types of drying had moisture contents ≤ 12%, even where the filaments had been allowed to stand for moisture equilibration.

The results from this trial are illustrated in **figure 3-24**, where the contents of the components are shown as mean values for the saffron products from the 10 sampling days. In comparison to the current commercial method, the high temperature method resulted in saffron with significantly more safranal (≈ 4 fold) and greater retention and/or availability of crocins. These results confirm the finding of the laboratory experiments (see chapters 3.B and 3.C).

It was observed however, that the oven dried saffron remained more brittle than that from the food dryer, even after equilibration time sufficient to return the moisture content to approximately that of from the food dryer (10-12%).

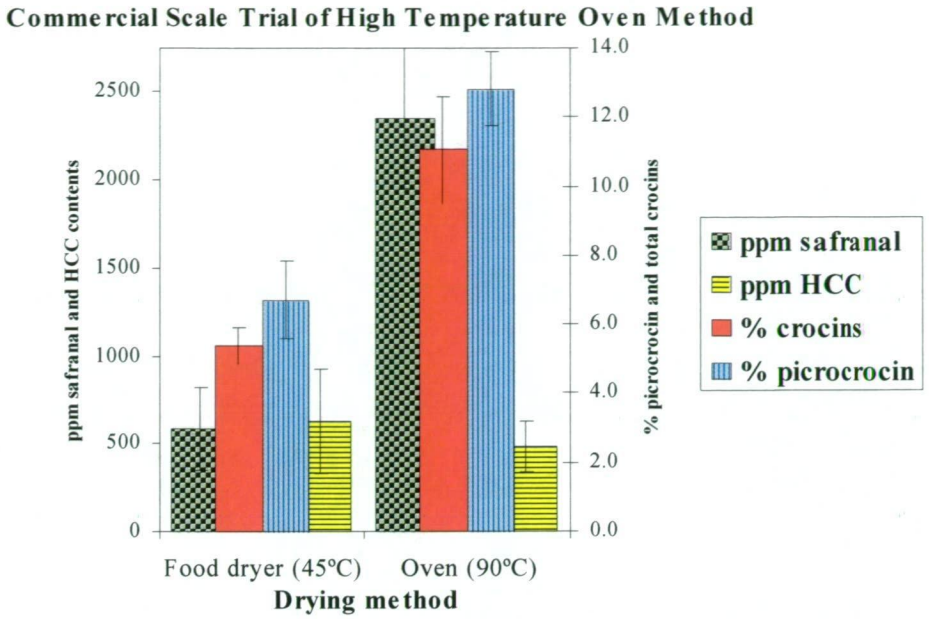


Figure 3-24: Mean composition of saffron from commercial scale trial of high temperature oven drying method compared to current (food dryer) method

3.F.2 High temperature/humidity method

Experimental

The same (*Thermoline*) oven that was used for the laboratory experiments was transferred to 1 of 3 farms on each of 4 days for the drying trials. Half of the total crop of flowers harvested each day was dried in the Grower's food dryer according to the normal commercial method (≈ 45 °C for 60 min.) and the remaining half dried in the humidified oven set at 90 °C for approximately 90 min. The oven was humidified with a dish of hot water as for the experiments described in chapter 3.D and the exact time of treatment was determined by the temperature of stigmas (measured as in chapter 3.D.2) reaching 90 °C indicating complete drying (see **figure 3-22**). This varied between runs from 80-95 min.

Sub-samples of approximately 500 mg of saffron from both these treatments were split into 2 replicates for later extraction and analysis (as for preceding laboratory experiments) described in General Methodology (2.A).

Results and discussion

The number of flowers harvested on each of the 4 days ranged between approximately 500 and 1200 so that the number of stigmas used per repetition of the drying treatments was between 250 and 600. The moisture contents of the 4 replicate saffron samples from the humidified oven drying were all < 12%, but the moisture contents of 2 of the 4 replicates from the food dryer treatment were above 12% (15 & 19 %). The moisture contents of these 2 samples were reduced to < 12% by drying in a food dryer for a further 30 min. at 40 °C in the laboratory 24 hours later.

The results from the saffron component analysis are shown in **figure 3-25** as the means from the 4 replicate treatments. The high temperature/humidity oven method produced saffron with significantly more safranal than the commercial method (approximately 8 fold). The effect of under-drying of 2 of the food dryer samples is clearly evident in the high mean (and standard deviation) for HCC content.

The 24 hour period was sufficient to allow significant generation of HCC via β-glucosidase activity, despite the samples being stored at 5 °C for most of this time. The inclusion of the samples that were initially underdried makes the food dryer samples not wholly representative of the commercial method properly applied. However, the greater generation of HCC might be expected to increase the safranal content (once dried to <12%) rather than prevent safranal production. This further emphasises the much greater safranal generation in the humidified oven. It is perhaps interesting to note that further drying did not result in wholesale conversion of HCC to safranal.

As for the high temperature method, the humidified oven method resulted in significantly greater retention and/or availability of crocins. The theory[*28, 32*] that fast drying (from high temperature and/or airflow) drying directly affects pigment availability by increasing the porosity of saffron filaments remains a possible explanation for this. However, the inclusion of added humidity to slow the drying process would seem to count against the theory unless the changes in porosity are not simply due to speed of drying, but also changes in membranes and cell structure that occur at the higher temperature irrespective of rate of moisture loss.

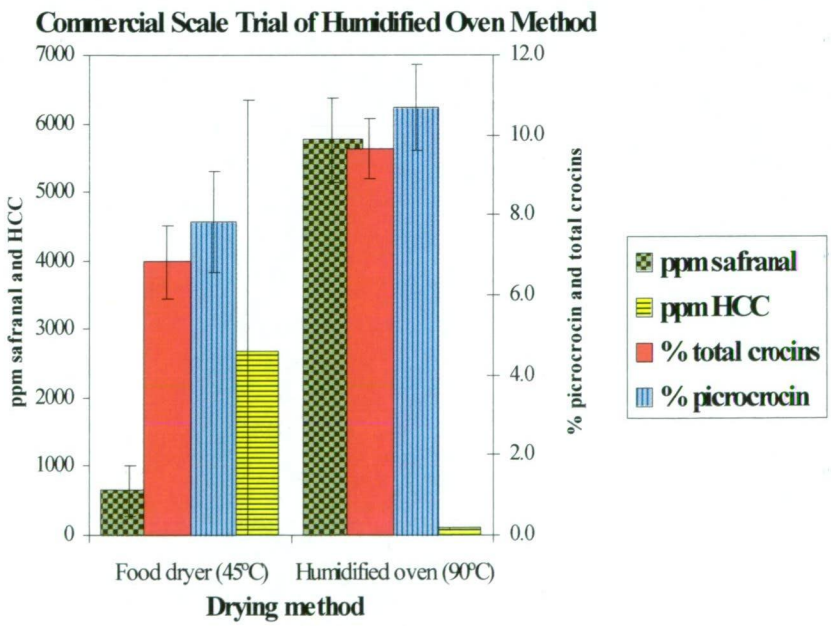


Figure 3-25: Mean composition of saffron from commercial scale trial of humidified oven drying method compared to current (food dryer) method.

Implications

The commercial scale trials confirm that the high temperature/humidity drying method produces saffron of superior quality (as measured by safranal and pigment content) and that it is possible to successfully transfer this method to a commercial scale. Notwithstanding this, adjustments would need to be made to accommodate larger numbers of stigmas than dried in these trials (600 stigmas). In transferring the method to a larger oven, the heating

characteristics of the drying chamber with any given mass of stigmas and surface area of hot water would need to be ascertained so that stigmas are dried sufficiently but not left at 90 °C for longer than necessary.

The matter of whether the measurement of higher pigment levels (in saffron dried by the new method) is wholly due to actual content in the stigmas, or is (at least in part) a result of greater availability stemming from structural or porosity effects, remains undecided.

3.G Micro-structural effects of drying in relation to porosity and pigment extraction

Introduction

In the preceding chapters, higher levels of crocin pigments were consistently measured in saffron dried using either the high temperature or high temperature/humidity methods (compared to the lower temperature commercial method). According to the theory proposed by Carmona *et al[28, 32]*, the a faster rate of saffron drying, then more porous the filaments as cells do not shrink together uniformly, and this results in higher colour extraction and thus measurement. However, this theory does not explain the higher pigment contents from the high temperature/humidity treatments where drying is slowed by the humidity applied (drying times being comparable to the low temperature food dryer method).

The aim of the following work was to determine if differences intercellular structure could be discerned from scanning electron microscopic (SEM) observation of saffron from different drying methods. Pigment dissolution rate and porosity were then quantified to see if these measurements correlate with structural observations.

3.G.1 Scanning electron microscopy

Experimental

Filaments of saffron from different drying treatments applied in the preceding chapters and fresh stigmas were cut into 5 mm sections and affixed to stubs. Besides examination of fresh stigmas, the, drying treatments used included: oven at 40 °C (without airflow), the current commercial method (food dryer at 40 °C with airflow), oven at 90 °C (without airflow) and humidified oven at 90 °C (without airflow). The final RH during the humidified oven treatment was approximately 50%. The drying times for these were 120, 90, 30, and 85 min. respectively. Fresh flowers were picked on the morning of the examination and transported intact in sealed containers at 4 °C until removal and sectioning of stigmas. Three sections were cut from each saffron type; one each from the stigma end, middle and at the junction with the pistil.

Scanning electron microscopy was performed on uncoated specimens using a FEI Quanta 600 environmental scanning electron microscope with an acceleration voltage of 15 kV and water vapour as residual gas atmosphere in the sample chamber at pressures of 0.8 to 5 torr. The images were acquired using FEI gaseous secondary electron or large field secondary electron detectors. In the SEM chamber the samples were cooled to temperatures of 1 to 5 degrees centigrade using a Peltier cooling stage

Results and discussion

Three images of saffron filaments from each of the drying treatments are shown in **figures 3-(26-30)** to illustrate the comparative structural differences.

The fresh stigmas (**figure 3-26**) exhibited relatively smooth and rounded cylindrical cells with only small intercellular spaces between them and no rupturing of the surface. The rounded protrusions that are clearly evident have been shown in previously reported SEM images of saffron stigmas but were not identified[*32*]. These protrusions may be caused by internal organelles such as cell nuclei or chromoplasts. The latter is likely as these are relatively large in Crocus stigma cells given the importance of pigment synthesis activity[*23, 178*].

The slowest dried saffron from the oven at 40 °C, without airflow (**figure 3-27**) appeared to have changed comparatively little from the fresh state; the major difference being cell shrinkage. That shrinkage appeared relatively even resulting in little or no increase in intercellular spacing and no surface rupturing. The protruding structures remained prominent.

The saffron dried in the food dryer (at 40 °C with airflow) showed a more pronounced shrinkage of cells giving an uneven appearance (**figure 3-28**). The surface was less even with ridges and grooves and a noticeable increase in intercellular spacing, but no visible rupturing. Some of the protruding structures remained prominent but not in all cells.

The most rapidly dried saffron dried (from the oven at 90 °C) exhibited no more cells shrinkage or ridging than above, but did show an apparent fusing effect between cells suggesting a breakdown of surface cuticular wax structure or cell wall membranes (**figure 3-29**). Moreover, distinct ruptures could be seen, usually at right angles (laterally) to the longitudinal direction of the cells. The protruding structures remained prominent in only some cells.

The saffron dried in the humidified oven at 90 °C showed a pronounced wrinkling of the cell surfaces with some fusing similar to the previous treatment (**figure 3-30**). Unlike the treatment above there was no lateral rupturing evident. The protruding structures were less prominent and could be seen in only some cells.

These observations support the proposition that different drying methods result in saffron with different structural characteristics; and that the more rapid the drying is, the more porous the structure resulting. Besides differences in intercellular spacing, this variation in porosity may be due to removal or disruption of the wax coating of the cuticle and/or cell wall or membrane breakdown. Such temperature related effects have been observed in cooked or dried tissues of other plant species subjected to temperatures of approximately 90 °C[*179-182*].

The crocin pigments are thought to be biosynthesised (from zeaxanthin) in the chromoplasts (plastids) with the final glycosylation step(s) being immediately followed by transfer via vesicles to the polar environment of the vacuole for storage[*23, 178*]. Any opening up of the intercellular structure allowing greater access of polar solvents to individual cells, combined with disruption of the wax, cuticle, and/or cell wall membranes of tissue beneath, would lead to increased diffusion of polar pigments from the cells into the surrounding solvent. This effect would be accentuated where the internal ultrastructure was also disturbed by heating, with effects such as rupturing of vacuoles further reducing the barrier to crocin diffusion from the cells.

Implications

At moderate temperature the degree of intercellular spacing and uneven cell shrinkage in saffron filaments appears to increase with speed of drying (determined by the use of airflow). The use of higher temperature (90 °C +/- added humidity) does not seem to cause significantly greater intercellular spacing or uneven ridging but results in fracturing of the epidermal surface and changes to the outer layer of cells that appears as a form of fusing suggesting that cuticle and/or cell wall membrane disruption may have occurred. While these observations support the proposition that crocin availability varies with changes in porosity due to different drying conditions, proof would require quantification of the relative porosity of filaments from these treatments.

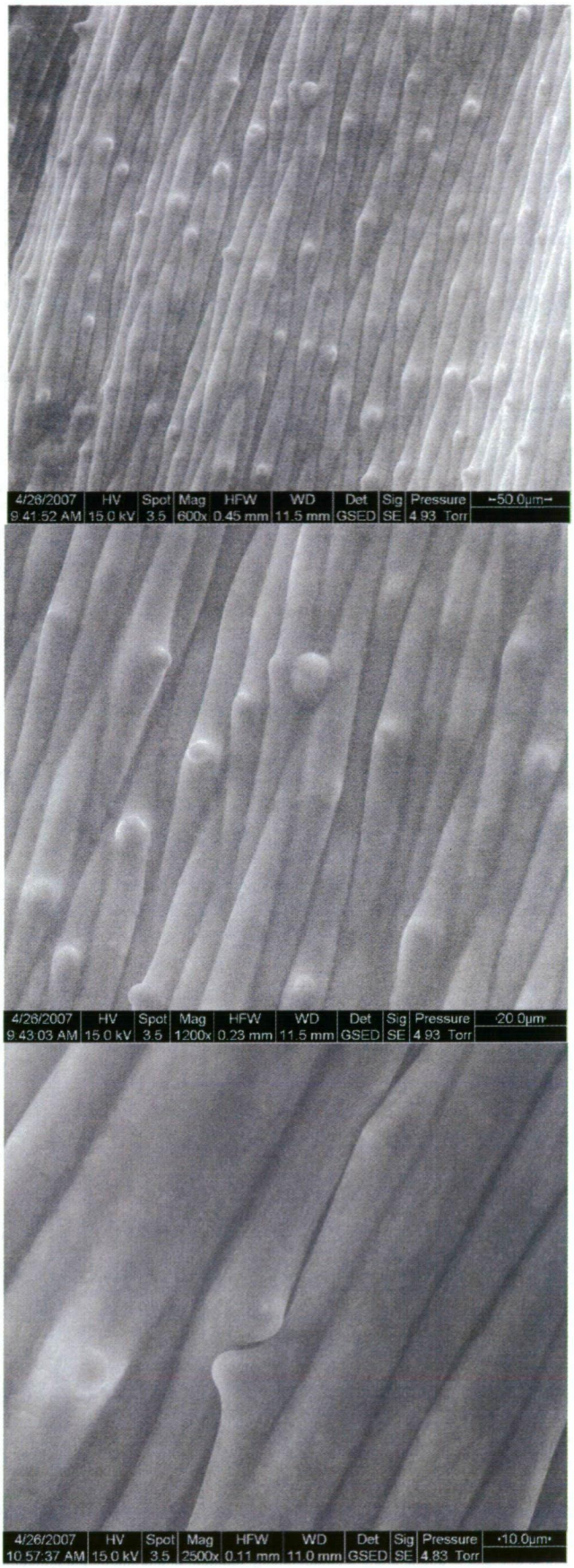


Figure 3-26: SEM views of filament surfaces of fresh (undried) stigmas.

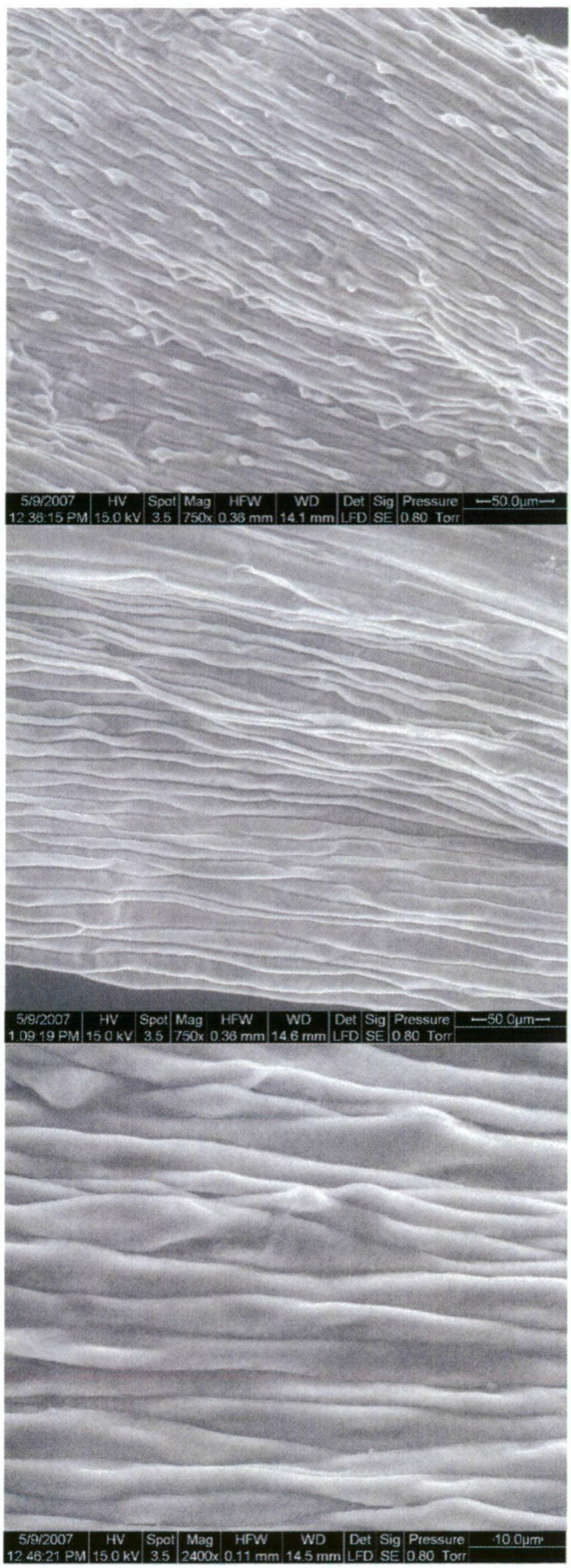


Figure 3-27: SEM views of filament surfaces of saffron dried slowly at 40 °C without airflow.

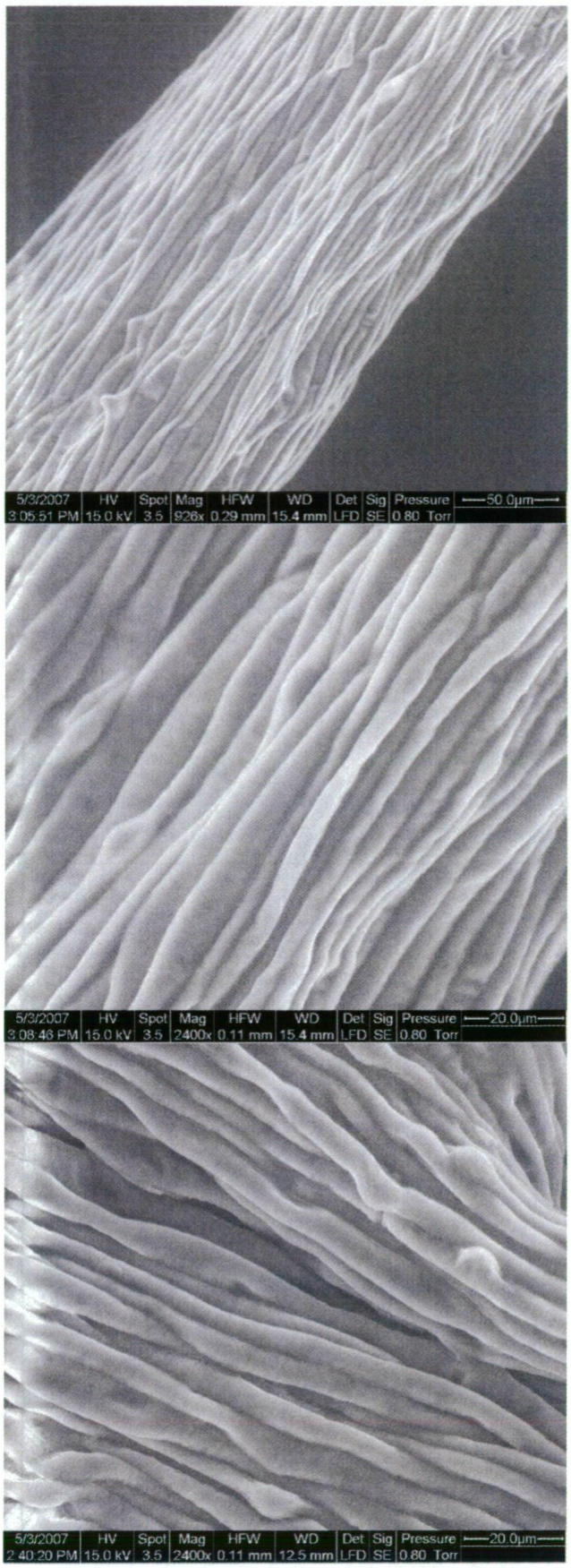


Figure 3-28: SEM views of filament surfaces of saffron dried in a food dryer at 40 °C with airflow.

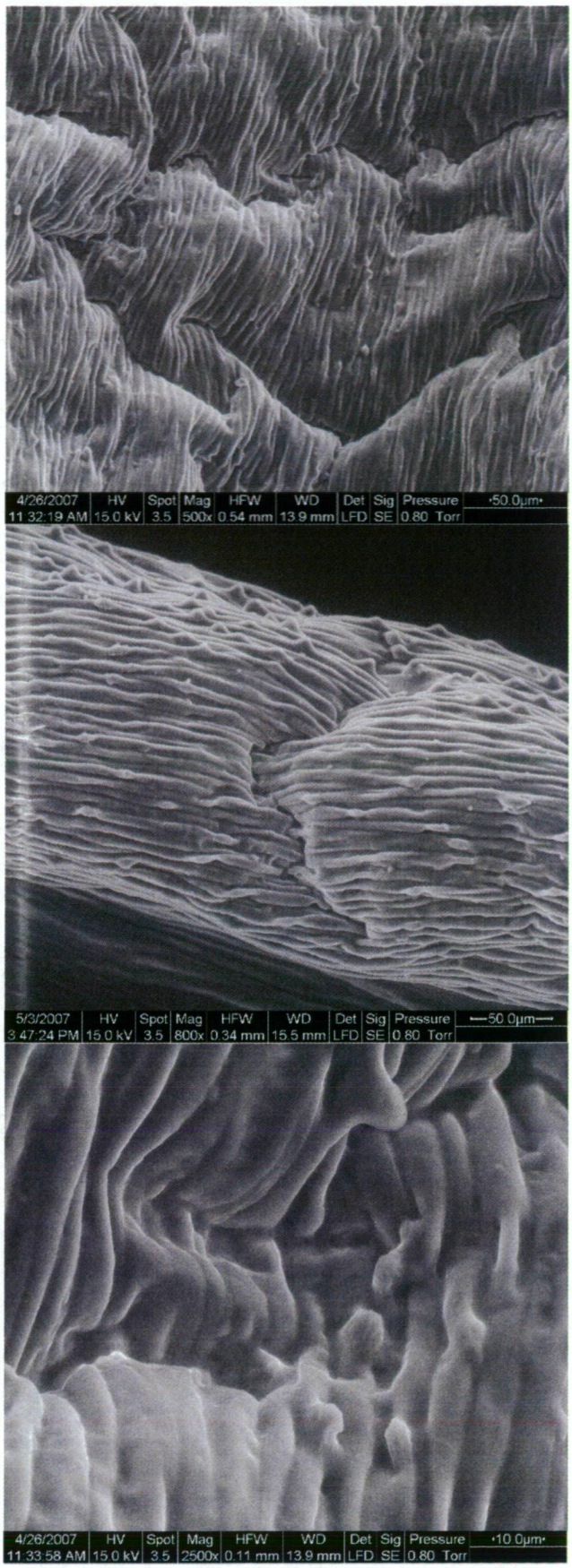


Figure 3-29: SEM views of filament surfaces of saffron dried rapidly in an oven at 90 °C without added humidity.

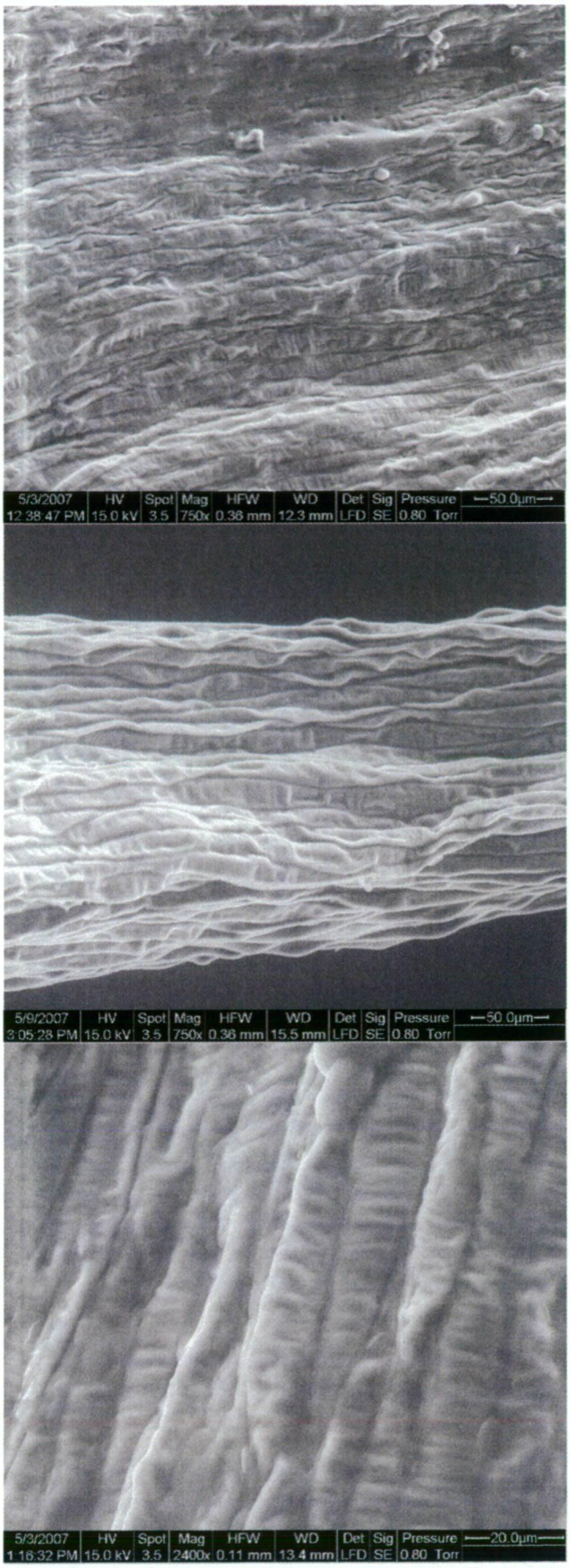


Figure 3-30: SEM views of filament surfaces of saffron dried slowly in an oven at 90 °C with added humidity

3.G.2 Effect of drying methods on the efficacy of pigment extraction from filaments

Introduction

It is apparent that different drying methods result in saffron from which the colour is released (into aqueous solution) at very different rates and that this may impact upon the any determination of colouring strength, however it is measured (i.e. ISO method or HPLC/UV). Carmona *et al* (1995)[*32*] have provided evidence for this being due to varying degrees of porosity or intercellular spacing resulting from different drying rates.

To test the effect of different drying methods on the rate and strength of colouring from intact saffron filaments, the dissolution of colour from a set mass of saffron filaments in a specified volume of water was measured by absorbance of the solution at 440 nm.

Experimental

The saffron used for the analysis was from the same drying treatments as used in the SEM studies: oven at 40 °C (without airflow), the current commercial method (food dryer at 40 °C with airflow), oven at 90 °C (without airflow), and humidified oven at 90 °C (without airflow). These involved drying times of 120, 90, 30, and 85 min. respectively.

Samples of 30.0 mg of intact filaments of saffron from each of the drying treatments were placed in a 400 ml beaker of distilled water on a magnetic stirrer set at 500 rpm. The beaker was covered in aluminium foil during each of the sampling runs to minimise photo-degradation of crocin pigments. Sub-samples of approximately 1 ml of the solution were taken at the time intervals for absorbance measurement at 440 nm before being returned to the beaker. This procedure was replicated 3 times for saffron from each drying treatment.

A 500 mg sample from each drying treatment was ground and the colouring strength measured according to the ISO method providing a theoretical measurement of "complete" pigment dissolution (assuming grinding allows this). The absorbance measurements of the intact filaments were then calculated as a proportion of the "complete" pigment dissolution absorbance to allow comparisons between samples with different total crocin contents.

Application of mathematical models was attempted in order to provide close fitting descriptions of the dissolution curves and thus theoretical time of full dissolution.

Results and discussion

Figure 3-31 shows the clear effects that drying different methods have on the colouring properties of intact saffron filaments. The more rapid the drying process used to dry the saffron, the more readily the pigments were dissolved and the more complete was the total dissolution. This supports the hypothesis that more rapid drying results in more porous saffron filaments. In theory, complete dissolution should occur given enough time, but this is not apparent above, possibly because the effect of pigment degradation becomes increasingly significant over time (and before complete dissolution occurs).

Figure 3-32 shows the fits for models applied to describe the curves. It was not possible to obtain close fits using the same model for all 4 plots. For the 3 slower drying treatments (oven at 40 °C, food dryer and humidified oven at 90 °C) the best fits were obtained using the Gompertz model for non-linear regression[*183, 184*] (RMSE values = 0.58, 1.24 and 1.88

respectively). However, this model gave a poor fit for the faster saffron dried in the oven at 90 °C (RSME = 5.22) for which the best fit found was the Gunary model (RSME = 1.20)[*185, 186*]. The Gompertz model does not give a time (X value) for the maximum Y value (or rather it is ∞), preventing a comparable numerical determination of the time of complete colour dissolution. Despite this, the application of different models is useful in that they are, in effect, statistical tests for the validity of the curves displayed.

To compare the relative colouring rates and strengths of the saffron treatments, the relative times taken to reach an arbitrary proportion of full colouring strength may be read from **figure 3-32**. If 70% of full colouring (from the ISO method) is used then the slowest dried (oven 40 °C) saffron took ≈ 10 hours to reach this point, the food dryer treatment took 5-6 hours, just over 2 hours for the humidified oven saffron and approximately 1 hour for the oven at 90 °C treatment. The maxima of % colouring strength reached by each treatment over based on the model curves were 71%, 75%, 93% and 92% respectively

The Gompertz model describes sigmoidal curves and fits most closely to the colour diffusion pattern from the 2 lower temperature treatments. This diffusion pattern suggests a possible hysteresis effect[*187, 188*], at least in the early stages when the dried tissue is absorbing water. This effect is much reduced or absent in the higher temperature treatments suggesting that whatever barrier is slowing water absorption (causing hysteresis) had been disrupted or removed by the higher temperature.

While the relative rate and completeness of pigment dissolution observed in these curves is in the same order as the treatment drying speeds, these differences are clearly not proportional. For instance; the difference in drying times between the food dryer and the humidified oven treatments was small (90 and 85 min. respectively), but the rate of pigment dissolution is substantially faster from saffron dried by the latter method; the curve for this being much closer to that from the (non-humidified) 90 °C oven. It seems likely that porosity, or at least pigment release is also affected by the maximum temperature reached, and possibly the duration, rather than by simply the rate of drying affecting uniformity of cell shrinkage. Changes in the epidermal cuticle or wax coating of the external surface of stigmas, or of cell membranes within the tissue, may occur at temperatures up to 90 °C resulting in greater access of solvent to pigments within[*75, 179, 180, 182, 189, 190*]. Such changes could be accentuated with the greater heat transfer occurring in a humid atmosphere despite stigma tissue shrinking more uniformly under such conditions producing less intercellular spacing.

Summary and Implications

The differences in colour dissolution between saffron drying treatments cannot be attributed to porosity alone, but may also be due to differences in pigment compartmentalisation and/or membrane integrity. In this respect tissue porosity (or intercellular spacing) must be distinguished from cell permeability[*191-193*].

Disruption of membranes may account for the absence of a hysteresis effect during the early stages of water absorption in saffron dried at the higher temperatures. Furthermore, the effect of different absolute concentrations of pigments in each of the saffron samples on the dissolution rates is not clearly separated from porosity or permeability effects. To provide a measure of solute movement and thus porosity independent from different endogenous concentrations or pigment compartmentalisation within cells, the movement of an exogenous solute both in and out of the stigma tissue would indicate the contribution of porosity to the differences in colour dissolution observed.

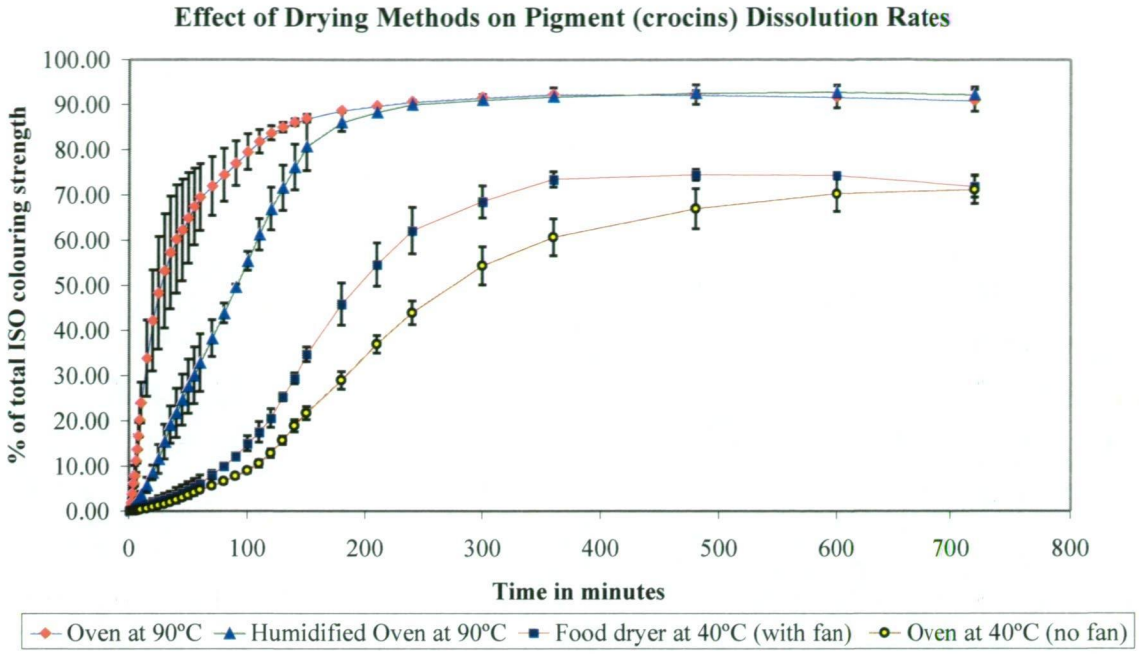


Figure 3-31: Plot of pigment dissolution rates of saffron from different drying methods

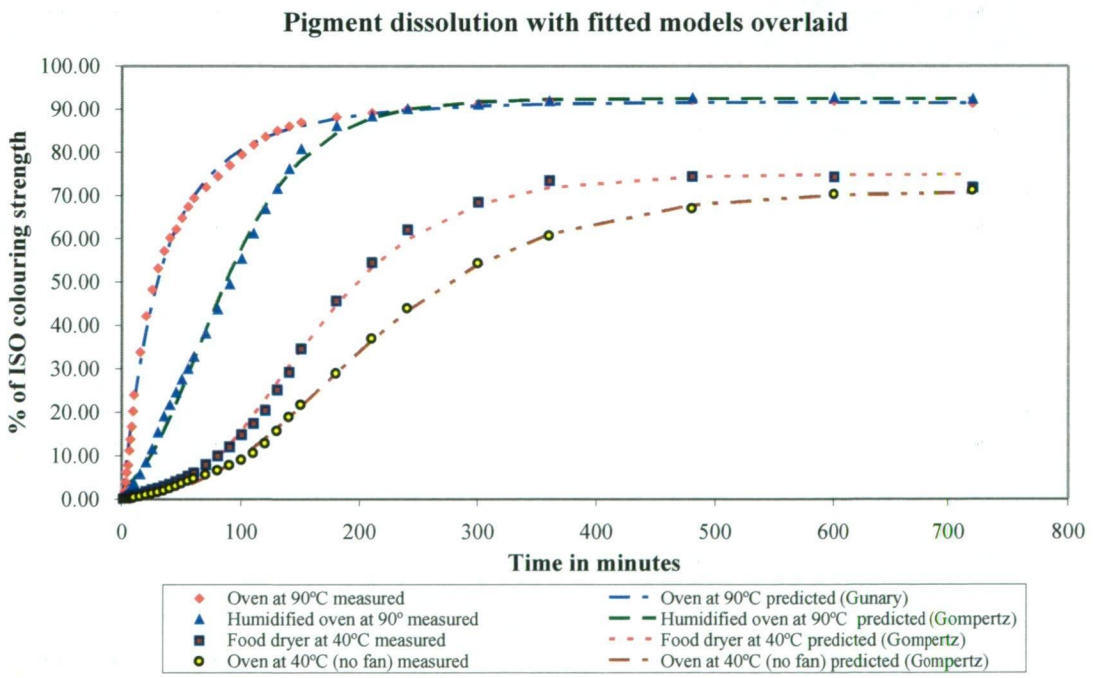


Figure 3-32: Plots of pigment dissolution rates with mathematical model regressions curve fits overlaid.

3.G.3 An alternative measure of relative porosity

Introduction

The aim of this experiment was to quantify the contribution of porosity to the observed differences in crocin pigment dissolution rates observed in saffron dried by different methods. By measuring the flux of small molecular weight ions both into and out of the saffron tissue, the relative porosity of samples may be quantified in isolation from the effects of different original pigment concentrations or effectiveness of membranes as barriers to movement of larger pigment molecules.

Experimental

Saffron samples used where from the drying treatments used for the preceding pigment dissolution experiment and the SEM study; oven at 40 °C (without airflow), the current commercial method (food dryer at 40 °C with airflow), oven at 90 °C (without airflow) and humidified oven at 90 °C (without airflow) giving drying times of 120, 90, 30, and 85 min. respectively.

Samples of 100 mg of stigmas contained within a muslin bag were soaked in a 1000 ppm solution of NaCl for 30 minutes. They were then spun by hand to remove excess fluid and rinsed in water 5 times, spun again and desorbed in 50 ml of water. The Sodium contents of the desorbing solutions were then measured at time intervals by flame photometry calibrated against a series of standard NaCl solutions. The procedure was repeated 3 times for each drying treatment.

Results and discussion

Figure 3-33 shows clear differences between treatments in the volume of salt desorbed and the rate of desorption. In order to calculate the time of full desorption non-linear regression models were applied to describe the desorption curves. The Gunary model[*185, 186*] was found to be the best fitting model available. **Figure 3-34** shows the lines determined by application of this model superimposed over the data points where the curves are described by the equation below (the constants A, B and C having been determined by regression analysis using this model in the SAS program):

$Y = X/ A + B * X + C\sqrt{X}$

Where X = time, Y = salt desorbed

Therefore to determine the time at which maximum salt had desorbed for each treatment:

$dY/dX = ((A+BX+ C\sqrt{X}) - X(B+½CX^{-½})/(A+BX+C\sqrt{X})^2$

When dX/dY is set to 0:

$X = 4A^2/C^2$

To determine the maximum Y (salt desorbed) value at this time:

$Y = X/(A+B(X) - C\sqrt{X})$

From the application of the model to data the following salt desorption maxima and times of full desorption were calculated for the 4 saffron drying treatments analysed (**Table 3-7**).

Table 3-7: Salt desorption rates and maxima in saffron dried by different methods calculated from the Gunary model regressions.

Saffron sample (drying treatment)	Desorbed salt maxima (ug/100 mg saffron)	Time of desorption maxima (min.)
Oven dried at 40°C	640	757
Food dryer at 40°C	655	187
Humidified oven at 90°C	706	174
Oven at 90°C	731	122

These results illustrate how different drying methods produce saffron filaments of varying porosity. More porous structure has resulted in greater uptake of Sodium during the absorption stage and then more rapid desorption. This movement would not be restricted by membrane permeability in the way that movement of larger polar molecules such as crocins would; and thus differences in desorption rates may be considered as directly due to differences in porosity[*191-194*]. The more the rapid the drying process, due either to elevated temperature or airflow, the greater the salt absorption/desorption, and thus the more porous the saffron.

The use of elevated humidity has had the effect of slowing the drying process and thus reducing the porosity. It is not clear as to whether the greater amounts of sodium desorbed in the faster drying treatments (more porous) is due to greater total capacity of the tissue or more rapid uptake during the absorption phase. A combination of both seems likely.

The small difference in drying time between the humidified oven and food dryer treatments is reflected in a proportionally small difference in maximum desorption time. This supports the proposition that this difference reflects the relative shrinkage rates of cells during drying. This proportionality is not apparent for the most slowly dried saffron (in the oven at 40 °C) that desorbed very slowly; the calculated time of full desorption being beyond 12 hours. This suggests that where membranes remain intact some other factor may be impacting on ion diffusion; possibly the Donnan effect[*191, 195*] which is known to cause retention of ions in cells with intact membranes.

Summary and implications

While the differences in the colour dissolution rate and strength of saffron from the different drying methods may be partly attributable to varying porosity (caused by relative uniformity of cell shrinkage during drying), such intercellular spacing does not provide the full explanation. The higher temperature (≈ 90 °C) methods produce filaments from which a greater amount of pigment is more rapidly extracted and this effect is greater than the porosity differences measured (by diffusion of small Na+ ions). The effect of humidity at the higher temperature is to slow drying and reduce porosity, but the pigment extraction from saffron produced this way remains much more rapid and complete than from the lower temperature methods. It is proposed that this is due to temperature related disruption of cell wall/membranes within the stigma tissue allowing greater movement of crocins out of the cells.

These findings have implications for the interpretation of both colour measurement by the ISO method and the findings of the drying experiments presented earlier. This work supports

the proposal of Carmona *et al* [*32*] that ISO measurements of colour strength give underestimates of total pigment content of slowly dried (at low temperature) saffron but expands on the their reasoning by including temperature related membrane disruption as a cause.

The ISO method employs grinding of saffron, which might be expected to reduce much of the variation in intercellular porosity, but may leave many cells intact so that membrane permeability differences remain significant. Moreover, most of the commercial demand is for saffron as whole filaments, which attract a higher price, and so there would seem to be a benefit in selling filaments from which greater colour is more rapidly obtained.

The greater contents of total crocins measured in saffron dried using the high temperature and high temperature/humidity treatments detailed in chapters 3B-3C and 3F (see **figures 3-9, 3-12, 3-16 and 3-24/25**) may have in part been due to underestimation of crocins from the low temperature treatments. This effect should, however, have been relatively small as all such samples were ground and extracted for 16 hours prior to HPLC/UV analysis and any pigment not extracted in this time frame could be considered "unavailable" in a commercial sense anyway. Furthermore, the finding that colour is extracted more slowly from filaments dried at high temperature with added humidity than without, supports the conclusion that the crocins are better retained by this method.

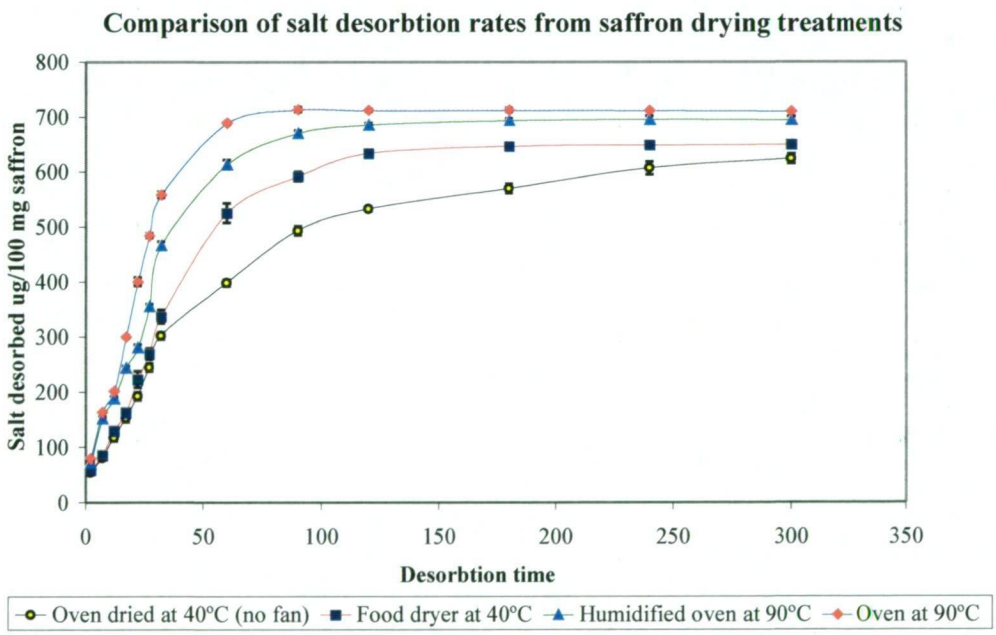


Figure 3-33: Plots of salt desorption for saffron dried using different drying methods

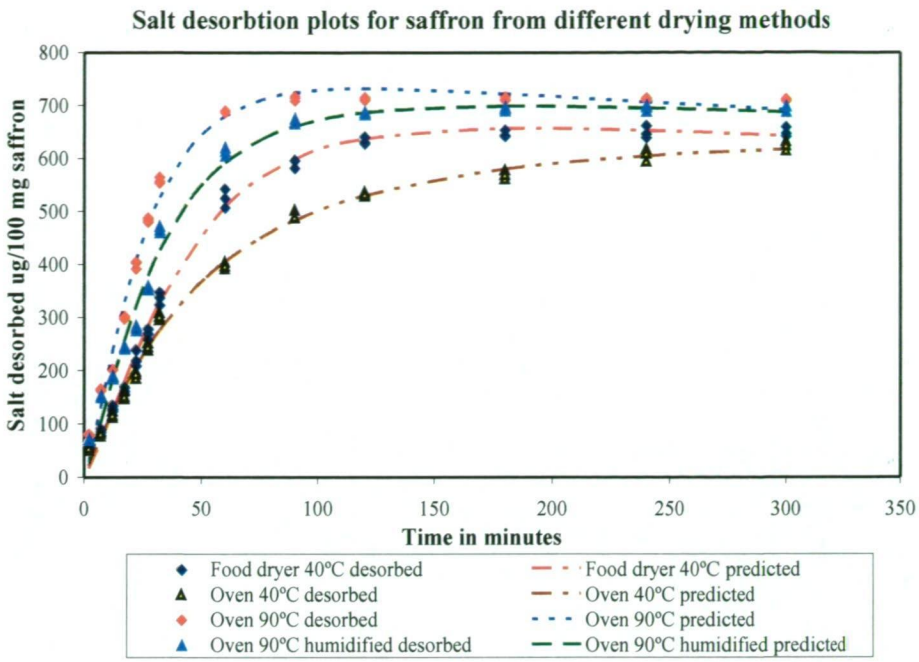


Figure 3-34: Plots of salt desorption with Gunary model non-linear regression curves overlaid

3.H Safranal production – enzymatic or direct hydrolysis?

Introduction

The findings detailed in chapter 3-D indicate that given specific drying conditions – high temperature (90 °C) at elevated humidity – significantly more safranal is formed from picrocrocin. However, if stigmas are dried to produce saffron with 7000 ppm safranal, this still represents only an approximate 15% yield or conversion rate (on a molar basis) if the original picrocrocin level is assumed to have been only 10% of dry Wt. (when the content in fresh stigmas may be up to 20%[*196, 197*] - see **figure 3-8**). Moreover, the reduction in picrocrocin levels in saffron after drying (typically reduced by ≥ 4% - **see figure 3-8**) does not match safranal gains irrespective of drying method. Clearly compounds other than safranal must be formed, as evaporation cannot be the major cause of the un-attributed picrocrocin loss that is not greater when high temperature drying is employed.

The plotting of stigma temperatures during high temperature and high temperature/humidity drying (see **figures 3-21&22**) also raises the possibility that safranal production during high temperature drying may be partly via enzymatic pathway if temperature is suppressed below the inactivation point of β-glucosidase. If this were a significant contributing factor to safranal production then it might be expected that drying (without added humidity) at approximately 60-70 °C would produce high safranal contents and this was not the case when tested in chapters 3.B and 3.C (see **figures 3-9 & 3-12**). However the possibility remains that high safranal production could be achieved by greatly enhancing enzyme activity.

The aim of the following experiments was to determine if more safranal could be produced by optimising conditions for β-glucosidase activity as an alternative to direct hydrolysis. This would be investigated by testing if more safranal could be generated in saffron filaments that had been previously dried; and if so, whether this further hydrolysis was enzymatic (via HCC) or direct. Additionally, by both artificially stimulating and inhibiting β-glucosidase activity in fresh stigmas before drying at either low or high temperatures, the relative contribution of the alternative pathways may be determined; as would be the potential for enhancing safranal production using enzyme treatments.

3.H.1 Re-hydration for safranal production

Experimental

Saffron samples from 3 distinct (original) drying treatments were selected for the re-hydration and re-drying experiment as follows:

A = Saffron filaments dried by the current commercial method - in a food dryer at 40 °C.

B = Saffron filaments dried in a food dryer at 40 °C, but only after an 8 hour delay between picking and drying; the resulting spice having reduced colouring strength (ISO absorbance strength = 172).

C = Saffron dried in the oven at 90 °C (without added humidity).

Replicate samples (x3) of approximately 1g were re-hydrated by placing them in open crucible dishes in a sealed container (a 5L vacuum desiccator) in the bottom of which a 1L dish of water had been placed achieving 100% relative humidity. This arrangement was left in the dark for 12 hours at 20 °C. The filaments were then re-dried in the crucibles using the humidified oven method (see chapter 3-D).

Sub-samples of approximately 300 mg from each replicate were taken before and after the moisture equilibration period and after re-drying. These were further split into 100 mg sub-samples for each of moisture determination, extraction then GC analysis (for safranal and HCC content) and HPLC analysis (for picrocrocin content).

Results and discussion

The re-hydration and re-drying treatment (using the humidified oven method) had distinctly different effects on the relative contents of safranal and its precursors depending on the original conditions of drying. Where the saffron had been firstly dried at moderate temperature (40 °C), significant β-glucosidase activity had occurred during the re-hydration (wet) phase as evidenced by the significantly elevated HCC contents shown in **Figure 3-35**.

In sample B, (which had initially been insufficiently dried), the higher initial HCC content indicates greater enzyme activity prior to the experiment, resulting in a smaller pool of picrocrocin and less HCC produced upon re-hydration. Although this sample initially had more safranal, the re-drying resulted in less safranal than in sample A where a greater pool of picrocrocin was available. Neither of these samples exhibited a safranal gain proportional to loss of HCC upon re-drying (for instance a 2200 ppm gain in safranal corresponded with a 5500 ppm (molar equivalent) loss of HCC) suggesting that either significant loss of safranal had occurred or that compounds other than safranal had been formed instead.

Where saffron, originally dried at high temperature (90 °C) was re-hydrated (sample C in **figure 3-35**), no significant production of HCC occurred, but significantly more safranal was then produced upon re-drying than in samples A & B. The conclusion is that the β-glucosidase in the stigmas tissue had been irreversibly inactivated by the original drying at 90 °C and thus further safranal generation was via direct hydrolysis rather than the enzymatic pathway via HCC. The magnitude of the safranal gain suggests that, in application of the high temperature/humidity method to fresh stigmas (see chapter 3.D), direct hydrolysis may account for most of the safranal generated (up to 7000 ppm) rather than a "burst" of enzyme activity during the temperature lag phase. Significant contribution of the enzymatic activity during the period of evaporative cooling cannot be ruled out, however, as the presence of small quantities of HCC indicated that some activity has occurred.

The significantly lower safranal contents in samples A and B after re-drying may seem surprising in that, despite having significantly lower picrocrocin contents before and particularly after re-hydration, more than enough remains to generate the quantity of safranal measured in C. This may be an important indication that the kinetics of the direct hydrolysis reaction are such that equilibrium between substrate and product may be important. If so, this would imply that the full conversion is not possible and constitute a limit to the potential for safranal generation.

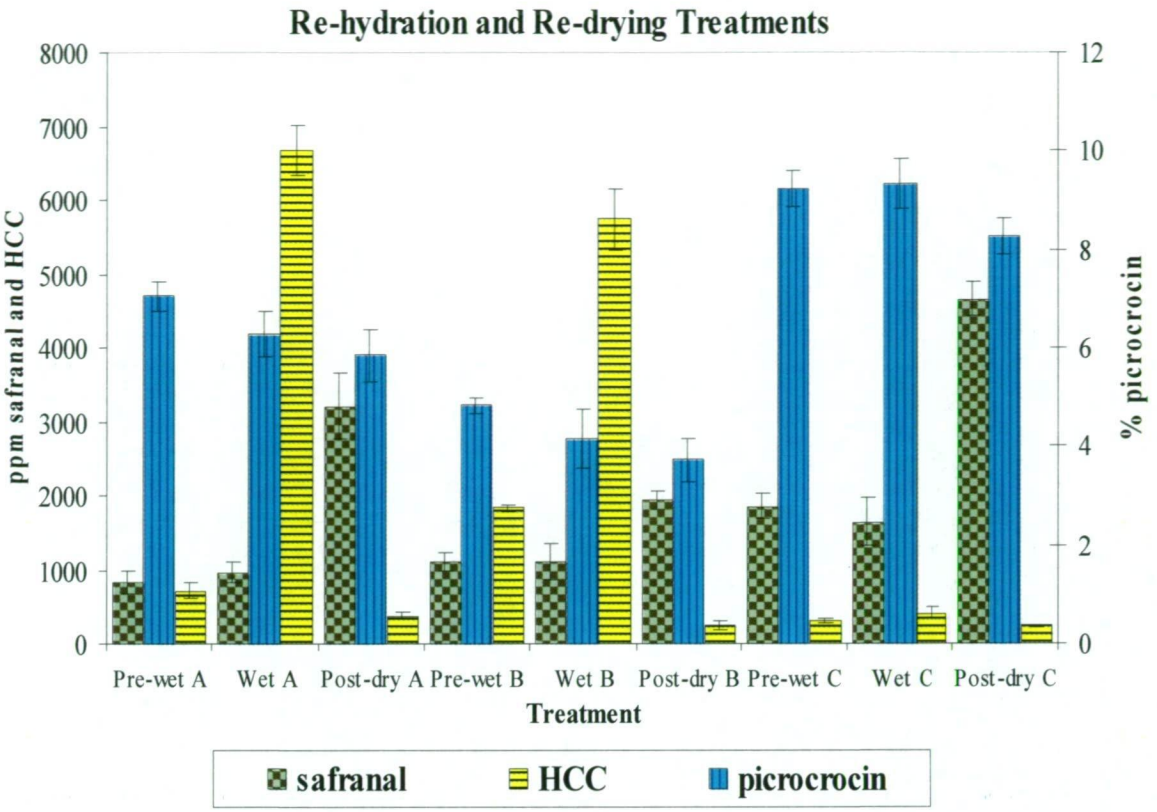


Figure 3-35: Contents of safranal, HCC and picrocrocin from re-hydration and re-drying treatment of saffron samples: where A = original drying in food dryer at 40 °C, B = original drying in food dryer at 40 °C but after 8 hour delay from time of picking and C = original drying in oven at 90 °C.

3.H.2 Addition of β-glucosidase

Introduction

The aim of the following experiment was to determine if greatly increased β-glucosidase activity from addition of the enzyme to stigmas would result in more complete conversion of picrocrocin; and if so, whether drying (under any of a range of conditions) would then result in safranal production comparable to or exceeding that produced by the humidified oven method.

Experimental

Stigmas were separated from flowers (picked in the same harvest) and frozen in sealed vials for storage prior to the experiment. Triplicate samples of approximately 200 mg of intact filaments for each of 8 treatment combinations were weighed into 20 ml vials immediately after removal from the freezer.

To each of these 1 ml of either, citric phosphate buffer (pH 5) containing 15 mg/ml β-glucosidase (Sigma Chemical Company; obtained from almonds; 6.9 units/mg) or only buffer

(controls) were added to the vials. Given this activity strength, the amount of enzyme added was calculated to be in excess of that required to hydrolyse all of the picrocrocin in the samples even assuming a 20% content in the stigmas prior to incubation[*198*]. These vials were then sealed, stirred with a vortex agitator and incubated at 40 °C for 6 hours. The filaments (still in the vials) were then subjected to 1 of 4 drying treatments: undried, dried at 40 °C in an oven, dried at 90 °C in an oven and dried at 90 °C in a humidified oven.

The enzyme treatments/assays were not blended as the stigmas having been frozen and then thawed had already undergone sufficient tissue/membrane disruption to allow ample free movement of substrates and enzymes.

Following drying, each replicate sample was split into 3 sub-samples; one each for dry Wt. determination, hexane extraction for GC/FID and GC/MS analysis and methanol extraction for HPLC/UV-vis analysis (as described in the General Methodology/Chapter 2).

Results and discussion

The results shown in **figure 3-36** illustrate the effect on picrocrocin hydrolysis from incubation of stigmas with additional β-glucosidase. Considerable generation of HCC from enzymatic hydrolysis of picrocrocin had occurred in the filaments of the control treatments as activity would have been enhanced by the freeze/ thaw process disrupting membranes and allowing greater substrate/enzyme contact (NB the scale of the HCC is 10 x that of the safranal in figure 3-35). This effect was observed in the initial drying studies (chapter 3.B) with treatment E in figure 3-9. The almost complete consumption of picrocrocin, resulting in a proportional (molar equivalent) increase in HCC, is shown from the undried enzyme added treatment.

The greater accumulation of HCC, from enzyme addition, did not necessarily result in greater safranal generation upon drying by the 3 methods used. The enzyme added samples did produce significantly more safranal than the control equivalents when dried in the oven at both 40 °C and 90 °C, but not when dried at 90 °C in the humidified oven. As shown by the findings in Chapters 3.C & D, it should be accepted that a high proportion of the safranal production in the control samples dried at 90 °C (particularly with added humidity) is due to direct hydrolysis. However, where the picrocrocin supply had been almost exhausted (by adding enzyme) this contribution content would have been much reduced and safranal formed is more likely to have come from dehydration of HCC.

The significantly lower safranal production in the humidified/oven dried enzyme treatment indicates that dehydration of HCC to form safranal may not be favoured in these conditions. Moreover, the safranal gains in the added enzyme treatments represent no more than approximately 20 % of the molar equivalent of the observed loss of HCC upon drying. This suggests that either safranal is being lost, or that compounds other than safranal are formed from HCC and that such alternative products are favoured by high water activity (humidity) at elevated temperature. As no net loss of safranal had occurred in the equivalent control drying treatment it may be assumed that the latter explanation must be the case.

Cadwallader[*9*] and Tarantilis *et al*[*30, 31*] have both suggested that degradation of safranal and/or HCC may lead to production of isophorone related oxidation products but were not specific about what these might be. Carmona *et al[29],* as part of a review discussing the origins of saffron aroma components (from both drying and cooking) and the possibility that these (including safranal) may also be produced by an alternative pathway involving degradation of crocins at high temperature (>> 100 °C), have proposed that 2 alternative compounds may be formed from HCC under certain conditions. These compounds, 2,6,6-trimethyl-1,4-cyclo-hexadien-1-carboxaldehyde (an isomer of safranal) and 2-methylene-6,6-

dimethylcyclohex-3-ene-1-carboxaldehyde were shown to increase in abundance in re-toasted and then cooked (boiled) saffron, with the latter only appearing after this treatment. They suggested that the higher energy available during toasting and/or boiling promotes production of these components not normally favoured by dehydrating at moderate temperatures. It may be that drying at elevated temperature (90 °C) and humidity is sufficiently similar to the conditions involved in boiling to favour conversion of the large pool of HCC (**in figure 3-36**) to these rather than safranal.

While the GCMS analysis of these hexane extracts did not identify the proposed product(s) of HCC, it did reveal a peak at RT ≈ 14.9 min (labelled X in **figure 3-38/2&4**) that only appeared in the samples where enzyme activity (and thus HCC production) had been maximized and then dried. This conversion appeared to be almost complete where that drying was at 90 °C (+ humidity which reduces evaporative cooling). Peak X was not positively identified, but displayed the Mass Spectrum shown in **Figure 3-39**.

A thorough search of the MS data in the literature[*29, 31, 39-41, 44, 51, 68, 97, 166-168*] and in-house MS data libraries failed to identify peak X. From the mass spectrum it appeared that the compound has a MW of 184. This was confirmed by analysing the sample using a Kratos Concept ISQ (70 eV electron ionisation direct insertion probe at 200 °C). The only saffron compounds of this MW previously reported in the literature are 5-hydroxy-methyl-4,4,6-trimethyl-7-oxabicyclo-heptan-2-one[*40, 47*] and 4-hydroxy-2,2,6-trimethyl-5-oxo-cyclohexanecarboxaldehyde[*31*] but the mass spectra of these compounds did not match peak X.

A comparison of the mass spectrum of peak X with that of HCC (MW = 168) (**figure 3-39**) suggests that this unknown is indeed a derivative of HCC as the pattern of ions is almost identical up to *m/z* 125. The difference in the spectra above this mass implies that the unknown had formed by the simple addition of an oxygen atom (i.e. $C_{10}H_{16}O_3$) to give a MW of 184. Two possible structures seem likely to explain this: either the formation of an epoxide (as in the compounds cited above) or by the addition of an extra hydroxyl group.

Interestingly in **figure 3-38/2** where the addition of β-glucosidase maximized the release of aglycons, but without subsequent high temperature drying, many more volatile compounds are evident. This is consistent with the findings of Straubinger *et al* [*47*] who reported the array of such potential aroma precursors in saffron.

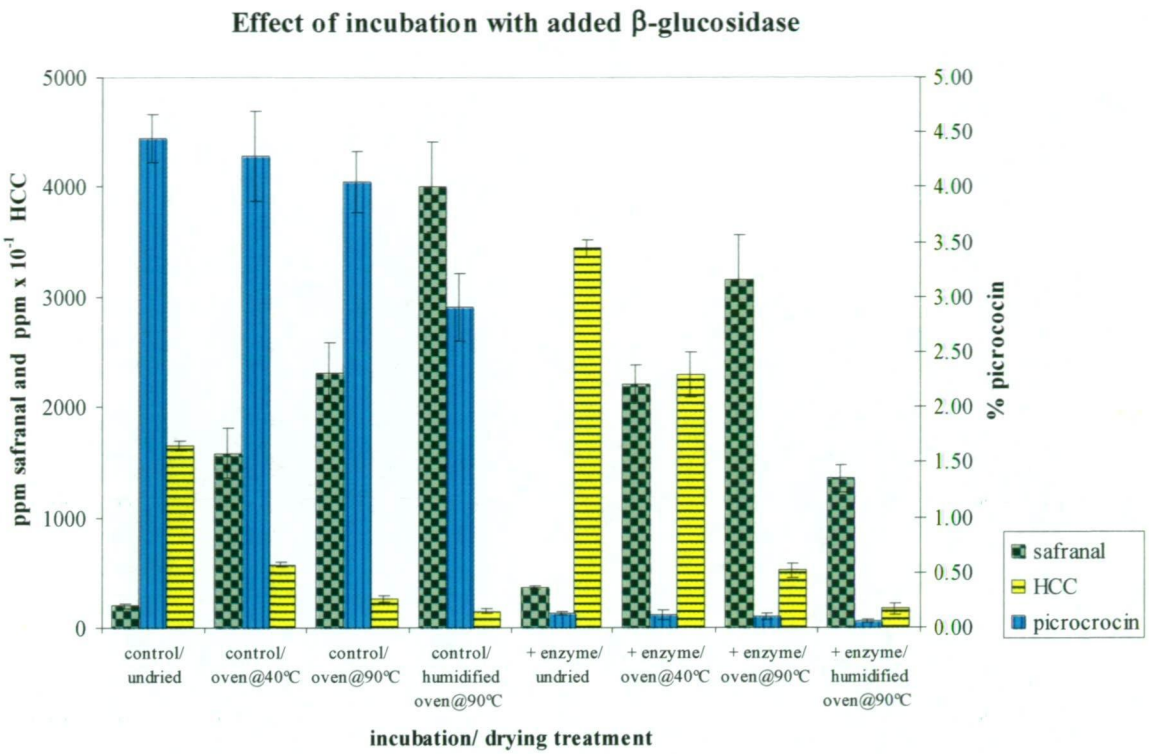


Figure 3-36: The effect of incubation with or without added β-glucosidase followed by different drying treatments on the contents of picrocrocin, HCC and safranal in intact saffron filaments. NB. While safranal and HCC contents are both given as ppm in this graph, the concentration of HCC plotted is reduced by a factor of 10 such that the highest level of HCC in this plot is approximately 10 x that of the highest level of safranal.

3.H.3 Effect of β-glucosidase inhibition

Introduction

By employing the same incubation treatments as used in the previous experiment, but with addition of a known inhibitor of β-glucosidase (nojirimycin [*199, 200*]) replacing the addition of β-glucosidase, the proportion of safranal production achieved by the direct hydrolysis (at high temperature/humidity) may be separated from conversion occurring due to enzymatic activity in the stigmas prior to drying and/or during the evaporative cooling induced, temperature lag phase of the of the high temperature methods. From this comparison, proof or otherwise of the primary contribution of the direct pathway during high temperature drying should be obtained. Moreover, such a comparison would give an indication of the importance of the relative size of the picrocrocin pool to the amount of safranal generated by the high temperature methods.

Experimental

Stigmas were separated from flowers (picked in the same harvest) and frozen in sealed vials for storage prior to the experiment. Triplicate samples of approximately 200 mg of intact filaments for each of the 8 treatment combinations were weighed into 20 ml vials immediately after removal from the freezer.

To each vial, 1 ml of either, citric phosphate buffer (pH 5) containing 100 ug/ml nojirimycin bisulphite (Sigma Chemicals) or just buffer (controls) were added. This concentration of nojirimycin bisulphite being approximately 10x that reported to cause a 50% inhibition in common β-glucosidases[*199, 200*]. These vials were then sealed and incubated at 40 °C for 6 hours. The filaments (still in the vials) were then subjected to 1 of 4 drying treatments: undried, dried at 40 °C in an oven, dried at 90 °C in an oven and dried at 90 °C in a humidified oven.

The enzyme treatments/assays were not blended as the stigmas having been frozen and then thawed had already undergone sufficient tissue/membrane disruption to allow ample free movement of substrates and enzymes.

Following drying, each replicate sample was split into 3 sub-samples; one each for dry Wt. determination, hexane extraction for GC analysis and methanol extraction for HPLC/UV-vis analysis (as described in the General Methodology/Chapter 2).

Results and discussion

As in the previous experiment, the incubation of stigmas with only buffer added (controls) resulted in significant but incomplete (≈ 50%) hydrolysis of picrocrocin to form the intermediate HCC (**figure 3-37 & 3-38b**). Again this pool of HCC was reduced upon drying, but not with a proportional increase in safranal contents; the highest level of safranal achieved (amongst the controls) resulting from high temperature/humidity drying where direct conversion would have been predominant.

The addition of buffer containing the inhibitor was seen to significantly reduce hydrolysis of picrocrocin compared to the control. A considerable quantity of HCC was still produced however, as some enzymatic hydrolysis would have occurred prior to infiltration of the inhibitor solution, particularly after thawing of the frozen tissue when internal membrane disruption would have resulted in accelerated enzyme activity.

The drying of stigmas (with inhibitor) at moderate temperature (40 °C) resulted in significantly less safranal formation than in the equivalent control treatment; a result that might be expected given that significantly less HCC was available. In the treatments dried at high temperature however, significantly more safranal was produced in the inhibited treatments (illustrated in **figure 3-38a**) compared to the controls, particularly in the humidified oven treatment where a safranal concentration (≈ 12000 ppm) larger than in all previous drying treatments was achieved. Clearly the greater pool of picrocrocin remaining as a result of enzyme inhibition during incubation allowed greater safranal generation via the direct pathway.

This result confirms that during high temperature/humidity drying, the overwhelming source of safranal is from direct conversion rather than via enzymatic activity occurring during the temperature lag phase. Moreover, it highlights the importance of the concentration of available picrocrocin to the kinetics of safranal production via the direct pathway.

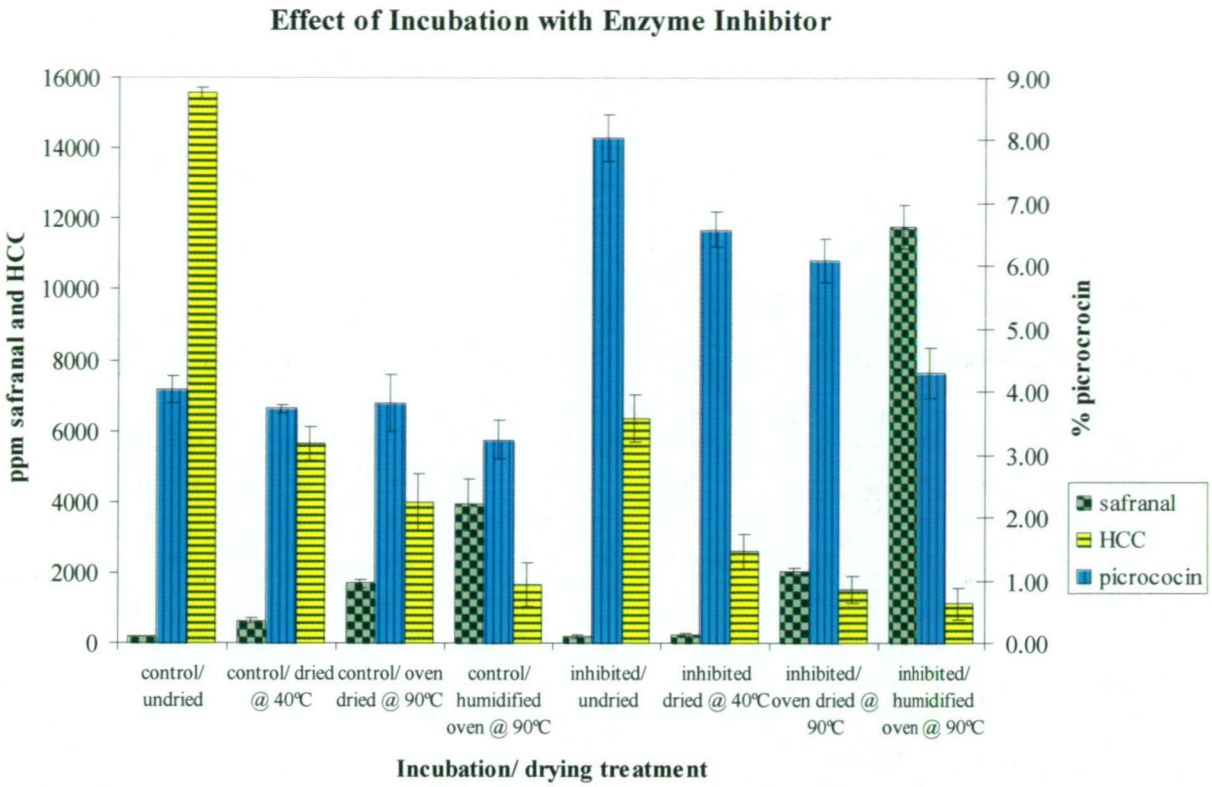


Figure 3-37: The effect of incubation with or without addition of β-glucosidase inhibitor, nojirimycin, followed by different drying treatments on the contents of picrocrocin, HCC and safranal in intact saffron filaments.

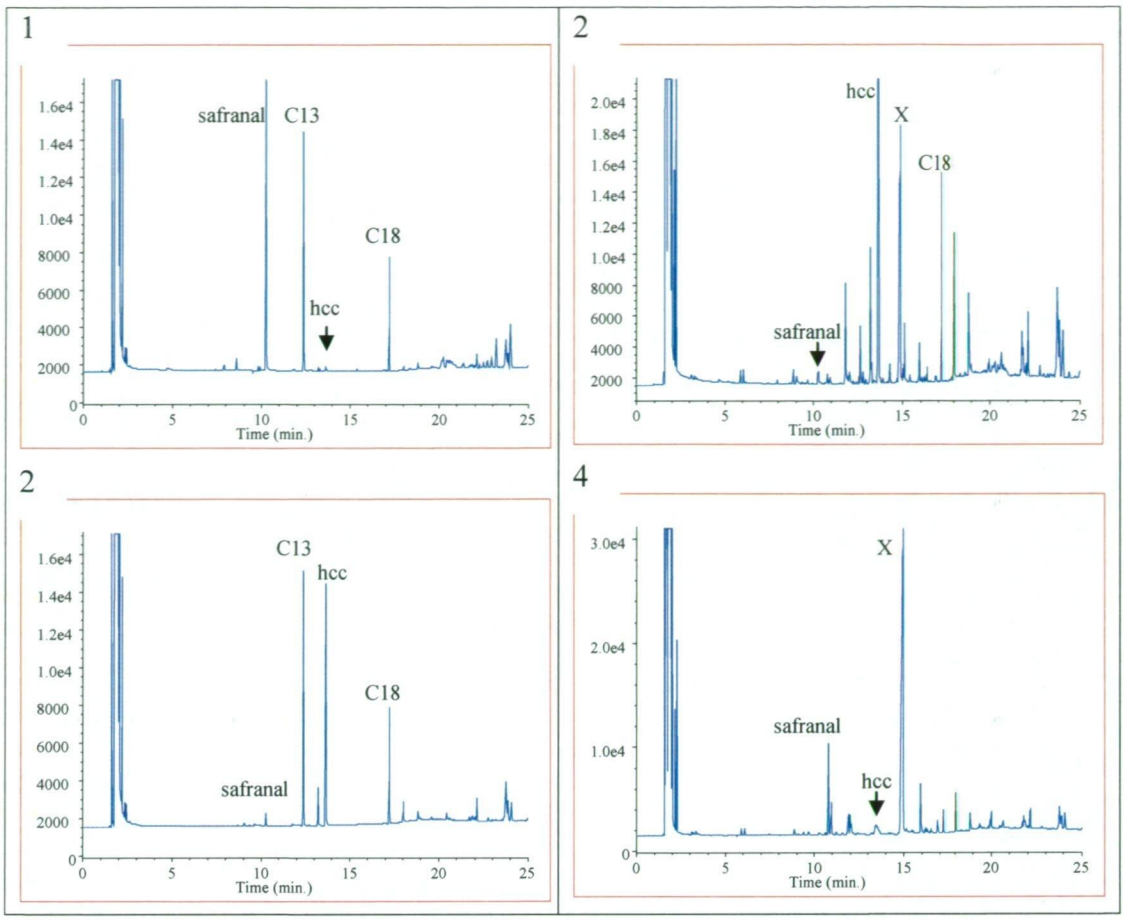


Figure 3-38: Gas chromatographs analysis examples of treatments from enzyme (β-glucosidase) addition and inhibitor addition experiments. 1= +inhibitor/humidified oven@ 90°C (see fig 3-38), 2= +control/undried (see fig 3-38), 3= +enzyme/undried (see figure 3-36), 4 =+enzyme/humidified oven@ 90°C (see fig 3-36). C13 & C18 are internal standard and X = unknown.

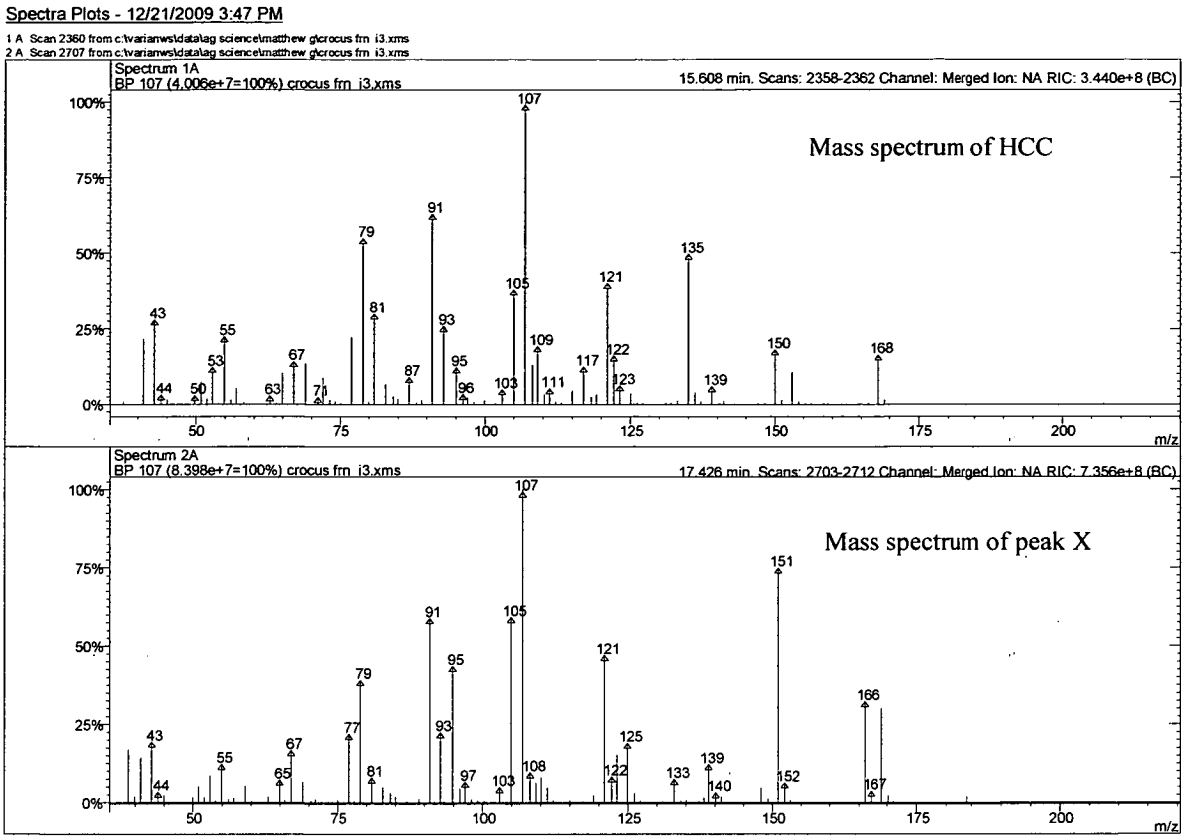


Figure 3-2: Mass spectra of HCC and unknown peak X from enzyme addition sample dried in the humidified oven @ 90°C (see figure 3-38 chromatogram 4).

3.H.4 Implications

While re-hydration/re-drying of previously dried saffron, and incubation of undried stigmas, especially with added β-glucosidase, may allow increased safranal production when dried at moderate temperature, the gains in safranal content are relatively modest and much less than the safranal content that may be produced by employing the high temperature/ humidity method. Also, given that incubation and drying slowly at moderate temperature results in significantly greater loss of (crocin) pigments (see Chapters 3.C&D) the advantages of the high temperature method are clear.

Furthermore, to optimise the potential for safranal production from high temperature/humidity drying, it is important to actually minimize enzymatic hydrolysis of picrocrocin prior to drying. This is because it appears that the formation of safranal from dehydration of the (intermediate) product of this activity, HCC, is not favoured at higher temperatures and rather other compounds are formed. Thus by maximising the pool of picrocrocin available, more safranal may be formed by direct conversion at high temperature/humidity.

4 PART B: WASTE FLOWER PRODUCTS

4.A Introduction

Pilot distillation of waste flowers, conducted before the start of this project, had resulted in product with an attractive floral and saffron aroma. These flowers had been stored initially at ambient temperature for approximately 24 hours, then at 4°C for 48 hours. The resulting distillate had a dry weight yield of 0.017% with a safranal concentration of 13%. It was thought that this sample was essentially stigma free and that the petals and stamens must contain a small but significant content of picrocrocin that yielded safranal upon heating.

The aim of the following studies was to further investigate the potential for obtaining secondary products from the waste flower parts (after removal of stigmas for saffron production) by either or both distillation and solvent extraction. This was to include determination of yields and both subjective assessment of flavour and/or aroma properties and chemical characterisation of the products.

4.B Initial distillation trials

Experimental

Waste flowers from 2 separate growers were collected as fresh or frozen samples on various days as outlined in **Table 4-1**. These samples were distilled and products collected as detailed in General Methodology (chapter 2). The oil products were analysed by GC-FID and GC-MS as for previous saffron extracts except that splitless injections employed to achieve sufficient response for analysis, as the samples were very small and thus dilute.

Results and discussion

The results of distillations conducted using waste flower samples from the 2006 harvest and the pre distillation treatments are shown in **Table 4-1**. From these it is evident that, where stigmas were rigorously excluded from the samples (8/April), no safranal was detected, and it is therefore concluded that the flowers distilled in the pilot study had contained some rejected stigmas (as they can be hard to see if the flowers are not fully open). Of these distillates (8/April) the fresh, un-incubated sample had the best yield and most pleasant aroma, but of such low impact as to be commercially uninteresting at this yield.

The remaining distillation treatments were therefore conducted on samples from the normal harvesting process that did contain reject stigmas and this % content (w/w) was measured in each case by picking out the stigmas prior to treatment. Some samples were incubated in an attempt to mimic the storage procedure of the pilot distillation sample. Other treatments included a drying procedure to try and mimic that employed for saffron production and freezing as any commercial collection of waste flowers may require freezing. Of these,

the frozen then incubated sample of flowers from 30 April gave the highest yield, but with unpleasant aroma characters in the product. The sample with the most pleasant aroma was produced from fresh incubated flowers collected over 14-28 April that also had the second best yield and highest safranal level. This yield would only equate to approximately 50 ml of product per tonne of fresh waste flowers. The major components (>90%) of all the distillates were alkanes (light waxes) that did not contribute favourably to the aroma. A typical GC profile from a splitless injection (due to the very low yield of oil) is given in **Figure 4-1**.

Given that these trials were not able to match the yield, aroma and composition results of the previous pilot distillation and that a significant yield and aroma quality improvement on even this pilot result would be required to make distillation commercially viable, it was decided to concentrate on solvent extraction trials.

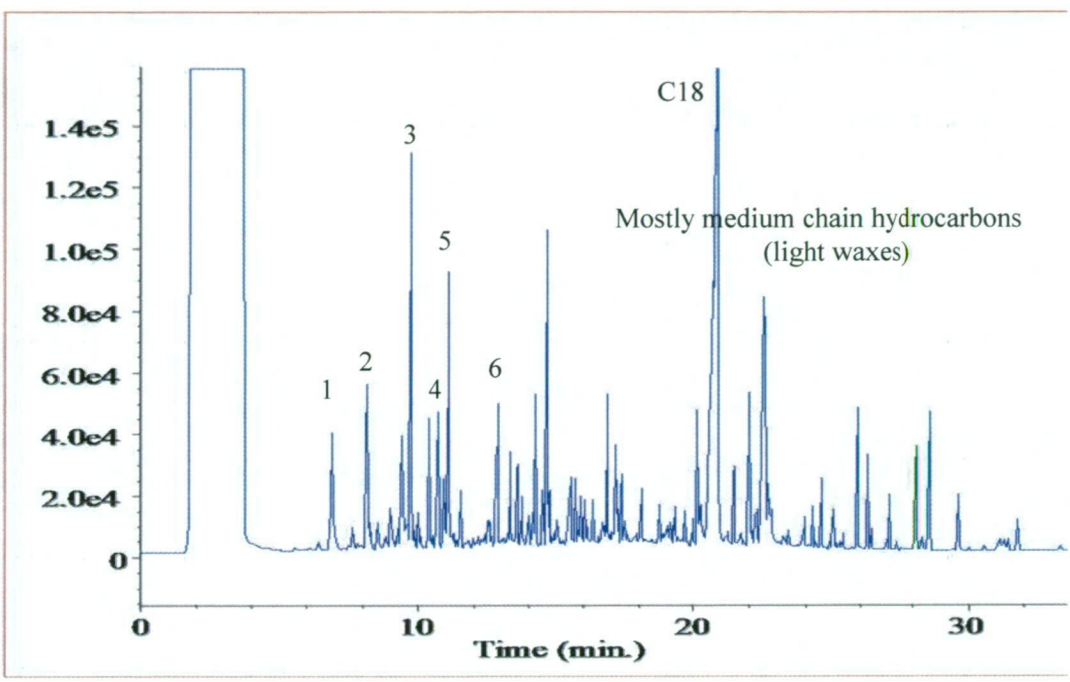


Figure 4-1: GC-FID chromatogram (splitless injection) of waste flower distillate. Peaks identified are 1 = linalool, 2 = phenylethyl alcohol, 3 = safranal, 4 = 2-hydroxy-3,5,5-trimethyl-2-cyclohexen-1,4-dione, 5 = phenylethyl acetate & 6 = 4-hydroxy-2,6,6-trimethyl-3-oxo-1-cyclohexene-1-carboxaldehyde.

Table 4-1: Waste flower distillation trials

Source and date of harvest	Sample Description	Pre-distillation treatment	% Moisture after treatment	% Yield of distillate (dry Wt.)	Components identified and % content in distillate	Organoleptic assessment
Grower 1 8/April	Waste flowers with stigmas excluded	Fresh/no incubation	91%	0.05%	Isophorone(t), phenylethyl alcohol(t), mostly light waxes.	Faint floral and waxy
Grower 1 8/April	Waste flowers with stigmas excluded	Incubated at 25°C for 24 hours	77%	0.03%	Light waxes	Waxy
Grower 1 8/April	Waste flowers with stigmas excluded	Incubated at 35°C for 24 hours	64%	0.02%	Light waxes	Waxy and slightly unpleasant
Grower 1 14-28/April	Fresh waste flowers with 0.25%(w/w) reject stigmas	72 Hours at 25°C/no further drying	31%	0.05%	Safranal(1.9%), HCC(t), isophorone(t), phenylethyl alcohol(t) and mostly light waxes.	Saffron, some floral and waxy
Grower 1 14-28/April	Fresh waste flowers with 0.25%(w/w) reject stigmas	72 Hours at 25°C then dried at 88°C for 60 min and 45°C for 24 hours	15%	0.03%	Safranal(0.05%) and mostly light waxes	Faint saffron but waxy and slightly unpleasant
Grower 2 30/April	Fresh waste flowers with 0.34%(w/w) reject stigmas	No incubation	88%	0.05%	Safranal(0.04%), HCC(t), isophorone(t), phenylethyl alcohol(t) and mostly light waxes.	Saffron, floral and waxy
Grower 2 30/April	Fresh waste flowers with 0.34%(w/w) reject stigmas	Incubated at 25°C for 48 hours	73%	0.04%	Safranal(0.02%), isophorone(t), and mostly light waxes	Some saffron but also unpleasant notes and waxy
Grower 2 30/April	Frozen waste flowers with 0.34%(w/w) reject stigmas	No incubation	81%	0.05%	Safranal(0.09%), HCC(t), isophorone(t), phenylethyl alcohol(t) and mostly light waxes.	Saffron and waxy
Grower 2 30/April	Frozen waste flowers with 0.34%(w/w) reject stigmas	Incubated at 25°C for 48 hours	70%	0.07%	Safranal(0.05%), isophorone(t), and mostly light waxes	Some saffron but also unpleasant notes and waxy

(t) = trace only (<0.01% of distillate)

4.C Initial extraction trials

Experimental

Solvent extraction trials were conducted on waste flower samples from the 2006 and 2007 harvests using a number of pre-extraction treatments including high moderate and ambient temperature drying/incubation methods (without airflow) and freezing. These treatments are detailed along with the results in **table 4-2** where the samples are divided into sets of treatments according to their source and harvest date. The stigma content of each sample (fresh or frozen) was determined by hand separation of these from the rest of the flowers prior to application of treatments. GC-FID analysis was used to determine the safranal and HCC contents of the products and to get an indication the relative level of other volatile components (not listed in the table 4-2).

Results and discussion

The extraction of samples where reject stigmas were excluded (8/4/06) resulted in products without safranal (**table 4-2**). The ethanol and hexane extracted undried samples from this sample set were re-dissolved in methanol and analysed by LCQ. No crocins or picrocrocin were detected in the ethanol extract (which should have been polar enough to extract them), confirming that the other flower parts are not a potential source of safranal. It was thought that the bright orange/red colour of the hexane extract might be due to fatty acid esters of crocins, but the LCQ analysis revealed that this colour was due to fatty acid esters of other unidentified carotenoids.

Pure ethanol extraction was rejected on the basis that it did not produce extracts with attractive colour, texture or aroma characteristics despite the higher yields achieved. The hexane extracts, although having very good colour and texture, and a pleasant fragrance, where not considered to have sufficient aroma impact. It was therefore decided to include rejected stigmas in all further trials and to measure the abundance of these as an estimation of the overall aroma contribution of stigmas to the products.

Freezing of the flowers prior to any drying treatment resulted in greater extract yields but poorer aroma quality (including lower safranal) than when fresh flowers were extracted. The greater yields would result from destruction of tissues and membranes allowing greater access of solvent to all the contents of the flowers. The loss of membranes and the difficulty with drying of the flowers after freezing and thawing (as they tended to clump together in a denser mass) are likely reasons for the lower safranal contents. This is consistent with the results of the freezing treatments applied to stigmas in the initial saffron drying experiments (see **figure 3-9**) where this disruption brought substrates and enzymes together resulting in an elevated HCC level, but did not favour the dehydration of HCC to form safranal.

Yields in each case were lower where undried flowers were extracted with hexane and this would have reflected the difficulty with partitioning where a significant content of water was present particularly where those flowers had been frozen and tended to form a slurry making it difficult to bring plant material and solvent together.

Drying at a moderate temperature in the oven produced more safranal and a better overall aroma than drying at higher temperature. The effect of drying temperature in this case appears to be the inverse of pure saffron (stigma) drying where the higher temperature treatments caused much greater safranal production. The explanation for this may lie in the much greater mass of plant matter being dried and the much slower rate of moisture loss by this mass. The

high temperature formation of safranal would appear to require concurrent moisture loss such that the plant tissue passes through an intermediate water activity range while at the appropriate temperature, which probably had not occurred with this larger mass.

The use of higher temperatures would also have resulted in the evaporative loss of other volatiles, further reducing the aroma content available for extraction. These products were observed to have less fresh, floral and green notes and also different background odours to the other hexane extracts, with woody, tobacco and earthy notes accompanying the base honey notes that all the extracts seemed to have to some degree. These were not as attractive as the fresher samples that had more top-note and floral odour impact.

The use of 10% ethanol in the hexane for extraction resulted in greater yields, but at the loss of the colour quality probably from extraction of other pigments contaminating the carotenoid esters. Sample 5a did have a very powerful aroma as it had the highest concentration of safranal, though this was from a flower sample much richer in stigmas than the others and the more delicate floral and honey notes were not evident in this product.

Ethanol was also used to effect an absolute conversion of this sample that, although resulting in the desired removal of waxes (46% w/w), also resulted in significant (73%) loss of safranal.

The best quality product was achieved where waste flowers were dried gently in the dark at ambient temperature for 3 days (sample 4b) and where gradual conversion of picrocrocin to safranal would have occurred without heat induced colour degradation or loss of other volatile top-notes. This was the highest yield of safranal per content of stigmas in the waste flowers. The proportion of reject stigmas in the flower sample was close to the average of 0.35% for all the samples used in the trials. On the basis of these results and observations, this product was chosen for further analysis and independent organoleptic assessment.

Table 4-2: Waste flower solvent extraction trials.

Sample, source and date of Harvest	Sample content	Pre-extraction treatment	% Moisture after treatment	Solvent used	% Yield of extract (dry Wt.)	Physical description	*Aroma rating	Safranal and *HCC* Contents of extract (ppm)
1a Grower 1 8/4/04	Fresh with stigmas excluded	Fresh/no drying	90%	Ethanol	5.10%	Dull reddish brown colour. Texture is viscous and glutinous	Poor	**Not detected**
1b Grower 1 8/4/04	Fresh with stigmas excluded	Fresh/no drying	90%	Hexane	2.17%	Bright orange red colour. Texture = soft concrete	Fair	**Not detected**
1c Grower 1 8/4/04	Fresh with stigmas excluded	Dried at 45°C for 24 hours	15%	Ethanol	4.41%	Darker red/brown colour. Texture = viscous and glutinous resin	Poor	**Not detected**
1d Grower 1 8/4/04	Fresh \with stigmas excluded	Dried at 45°C for 24 hours	15%	Hexane	2.33%	Orange red colour Texture = soft concrete	Fair	**Not detected**
2a Grower 1 16/4/04	Fresh with 0.27% stigmas	Dried at 45°C for 24 hours	14%	Hexane	2.39%	Orange red colour Texture = soft concrete	Good	**157 ppm** *74 ppm*
2b Grower 1 16/4/04	Frozen with 0.27% stigmas	Dried at 45°C for 24 hours	19%	Hexane	2.83%	Orange red colour Texture = soft concrete	Fair	**Trace only** *26 ppm*
3a Grower 2 14-28/4/04	Frozen with 0.24% stigmas	No drying	83%	Hexane	2.09%	Orange red colour Texture = soft concrete	Fair	**Trace only** *341 ppm*
3b Grower 2 14-28/4/04	Frozen with 0.24% stigmas	Dried at 45°C for 24 hours	20%	Hexane	2.91%	Orange red colour Texture = soft concrete	Fair	**23 ppm** *123 ppm*
3c Grower 2 14-28/4/04	Frozen with 0.24% stigmas	Dried at 88°C for 60 min and 45°C for 24 hours	16%	Hexane	2.89%	Dullish Orange red Texture = soft concrete	Fair	**Trace only** *46 ppm*
4a Grower 3 4/5/05	Fresh with 0.37% stigmas	Not dried	90%	Hexane	2.14%	Bright orange red colour. Texture = soft concrete	Good	**8 ppm** *35 ppm*
4b Grower 3 4/5/05	Fresh with 0.37% stigmas	Dried for 3 days at ambient (20°C)	31%	Hexane	2.73%	Bright orange red colour. Texture = soft concrete	Very good	**487 ppm** *534 ppm*
4c Grower 3 4/5/05	Fresh with 0.37% stigmas	Dried at 45°C for 24 hours	16%	Hexane	2.37%	Orange red colour Texture = soft concrete	Good	**274 ppm** *282 ppm*
4d Grower 3 4/5/05	Fresh with 0.37% stigmas	Dried at 90°C for 60 min then 45°C for 24 hours	11%	Hexane	2.15%	Dull Orange red Texture = soft concrete	Fair	**4 ppm** *51 ppm*
5a Grower 1 14-24/4/05	Frozen with 0.86% stigmas	Dried at 45°C for 24 hours	17%	Hexane/ ethanol (90:10)	5.16%	Dull reddish brown Texture = paste	Very good	**680 ppm** *211 ppm*
5b Grower 1 14-24/4/05	Frozen with 0.86% stigmas	Dried at 90°C for 60 min then 45°C for 24 hours	13%	Hexane/ ethanol (90:10)	4.75%	Dull reddish brown Texture = paste	Fair	**35 ppm** *Trace only*

* The aroma rating was given purely on the level of interest as a potential fragrance product and so is a combination of aroma quality and strength. For more detailed description of products see text.
Trace only = < 0.01%

4.D Independent Organoleptic Assessment of Extract

Experimental

The sub-sample of the 4b extract was sent to an agent in the United Kingdom who forwarded the sample to a leading perfumer, Dr J. Heffernan[*201*] for a description of the fragrance character and assessment of potential as a fragrance product.

Results and discussion

The following are Dr Heffernan's comments from an organoleptic assessment of the product:

"Quite green in dilution, vegetal, with a heavy floral back note but the green-ness is the defining factor. It has a sweet coumarin note to it as well'.

In concentration it has a floral top note - heavy floral, exotic, reminiscent of ylang ylang and jasmine. The top note isn't very 'toppy', the odour seems to kick in as a 'heart'. Exotic, narcotic flower and honey notes, and the green-ness subordinate. The green-ness is light and fresh, giving a fresh aspect to the product in concentration. Almost 'cucumbery' in its freshness, very nice.

Quite sweet and exotic in concentration. Very like French Narcissus absolute.

Market potential- small and niche. 'People would bite your hand off if it was the right price and available in quantity'. Price suggestion: several hundred £/kg would guarantee a market. You could approach SRS Aromatics, or another agent for florals to sell it. Is there a health aspect with saffron?

It could be used with synthetics to give a broader, more natural base to the perfume. In all-naturals it would give a green fresher sensation, which you could build other florals on top of, the saffron would be the heart of the floral."

Although this assessment is very much preliminary and from only one extract produced without any replication, the expert opinion above would suggest that there is a potential for a fragrance product from the waste saffron. Considerably more work was clearly required to isolate the aroma notes in this extract type and application of GC-organoleptic (sniffer-port) techniques would allow this with complementary GC-MS analysis providing the chemical identities.

4.E Composition and collection of waste flower parts

Experimental

To assess the practicality of commercial scale air-drying of waste flowers, samples were collected by growers on 4 farms during the 2006 harvest period. Drying was achieved by placing the flowers on shade cloth strung up as horizontal nets in sheds away from direct sunlight and wind.

Trial extractions of 3 sub-samples (of approximately 90g each) of the flowers were then performed with 3 proportions (0, 1% and 2% w/w) of saffron added (This product was rejected for sale due to colour degradation but had good aroma content = 1500 ppm safranal). This served as an estimation of the aroma content required to make the extract sufficiently attractive for commercial sale.

Results and discussion

Drying proved to be very slow, though it was noted that this harvest period was characterised by particularly cold and damp weather conditions (the coldest April on record for southern Tasmania). Despite this, the flowers were successfully dried without significant loss of petal or stamen colour even where drying down to <15 % moisture took up to 2 weeks. It proved necessary to only place the flowers in bags for storage after dryer (less humid) days or the flowers remained too moist.

A total weight of 4.1 kg of dried flowers was collected from the 4 farms and the mean residual moisture content of these was 13%. The mean stigma content of the flowers was measured at 0.17% w/w.

These extractions resulted in a mean product yield (of concrete) of 2.46% w/w for the three extracts. The products from the flower samples with 0, 1 and 2% added saffron were analysed by GC-FID and safranal concentrations of 0.16%, 0.64% and 0.89% w/w were measured in the extracts respectively.

Organoleptic assessment revealed that the sample without added saffron exhibited a very pleasant aroma with delicate floral and hay notes with a honey background but lacked impact. The 1% sample exhibited all the features above but with much stronger impact from the safranal giving the fragrance strong jasmine and violet notes. The safranal impact of the 2% sample was considered to be too strong in that much of the background floral and honey notes were "swamped". The 1% sample was therefore considered to be the best, though all three of these samples should to be submitted to independent analysis by Industry experts to determine the appropriate saffron/waste flower mix.

4.F Composition and properties of non-polar extract product

Experimental

The composition of an extract sample produced using exactly the method as for sample 4b, but without addition of stigmas (see Chapter 4-C) was investigated by HPLC-UV-Vis, HPLC-MS and GC-MS analyses, the conditions of which are detailed in the General Methodology (Chapter 2). For the former 2, samples of extract were dissolved in methanol/hexane (80:20), while for the latter samples were redissolved in chloroform. This extract was also compared to that from fresh (undried waste flowers to ascertain if drying had any effect on the pigment composition.

Where the conjugation with fatty acid esters was suspected these fatty acids were removed by a saponification procedure also detailed in chapter 2 and the sample was re-analysed.

A methanol extract of the same waste flower sample was also analysed as above for detection of any crocins present.

Results and discussion

No traces of crocin pigments were detected in the extract. This might be expected given that it had come from stigma free waste flowers and considering that the solvent was non-polar. However, Vignolini *et al* [*202*] have recently detected small amounts of crocins in similar extracts, though whether their flower samples were free from stigma "contamination" is uncertain. To help answer this question a sample of the methanol extract (used for chapter 4.F) was analysed and as no crocins were detected the finding above seem likely to have been a result of contamination.

The initial interpretation of the HPLC-MS analysis of a sub-sample of the extract apparently indicated that the pigments responsible for the bright orange/red colour are predominately fatty acid mono-esters of molecular wt. 550 carotenoids. This was deduced from the chromatogram of the absorbance data (at 440 nm) and the LC-MS chromatograms (see **figure 4-2**), which show a cluster of peaks with protonated molecules, observed at *m/z* 817, 815, 789, 761 and 733 consistent with parent MW. 550 carotenoids each conjugated with a fatty acid of various lengths. These would correspond to the stearate (C18:0), oleate (C18:1), palmitate (C16:0), myristate (C14:0) and laurate (C12:0) esters, a profile commonly reported in carotenoid esters from plants[*203-207*].

By plotting specific mass chromatograms, the patterns of peaks conjugated with each fatty acid are evident. Given that each of these peaks has the same parent molecule mass it is concluded that they are isomers of one another. The daughter ion at *m/z* 533 in **Figure 4-3** (of the MS/MS data of the peak at RT = 29 min.) seemed to correspond to the protonated dehydrated free carotenoid as it occurred regardless of fatty acid chain length.

There are several 550 Mol. Wt. carotenoids reported in the literature including anhydroluteins I, II and III, and crocoxanthin. Of these the first three have been reported from higher plants subjected to heat processing or from animal metabolism of ingested lutein, while the latter is only reported from algae[*208*].

However, a closer inspection of the data suggested another option. **Figure 4-4** shows the trace occurrence of an ion at *m/z* 1074 (magnified here x 5 compared to the other ions). This raises the

possibility that the *m/z* 550 ion is not the parent molecule mass of a mono-ester, but in fact the dehydrated parent molecule of a di-ester of lutein where the loss of both fatty acid groups during MS results effectively results in di-anhydro-lutein rather than the *m/z* 568 molecular ion of lutein. The predominant pattern of peaks with *m/z* ions between 732 and 816 in **Figure 4-2** would therefore be due to the loss of one fatty acid from each di-ester resulting in mono-esters during MS analysis. A closer inspection of the data (**figure 4-5**) also revealed trace signals for specific masses at *m/z* 1045 as well as 1074, both giving weak plots of peaks comparable to the overall pattern observed for UV and *m/z* 732-816 ions.

This interpretation is consistent with the results of Breithaupt *et al*[*205*] who, in analysing extracts of marigold, found that the ions attributable to the complete protonated di-ester molecules were not detected, but that each di-ester molecule had lost at least 1 of its 2 fatty acids during MS. **Figures 4-4&5** show that a trace response for the di-ester molecular ions can be detected. Breithaupt *et al*[*205*] found that the *m/z* 551 fragment ion $[M+H-FA)]^+$ only occurred with analysis of lutein monoesters and this ion is absent from the spectrum of these peaks (**figure 4-4**)

To better aid identification of these parent carotenoids, separation from the fatty acid esters by saponification was required. The product of this process was redissolved in methanol and analysed by HPLC-UV/Vis using a variety of solvent gradients and column configurations (described in General Methodology in chapter 2.B.) to facilitate separation of the carotenoid isomers. The initial solvent gradient used was the same as for the crude extract (mid polarity) and produced a single peak eluting early (as would be expected for relatively polar carotenoids such as those reported above). This and the mass spectrum of the main peak are shown in **Figure 4-6**, with the latter displaying a fragmentation pattern typical of free lutein with a small peak for the protonated molecular ion at *m/z* 569, the large dehydrated lutein peak at *m/z* 551 and the double dehydrated lutein ion at *m/z* 533. It is not consistent with the spectrum for any anhydrolutein isomer due to the presence of the *m/z* 569 ion.

However, his peak exhibited subtle variation in the absorbance spectrum across its width suggesting that it was composed of multiple compounds; and as the molecular mass of the peak remained constant, this suggests co-elution of isomers.

The use of a more polar solvent gradient delayed elution of the compounds and afforded some separation, but not enough to greatly aid identification. However when two C18 columns were linked in series using this more polar gradient, reasonable separation was achieved (**figure 4-7**) resulting in 2 significant peaks and at least 6 minor peaks.

The absorbance spectra of these peaks are given in **Figure 4-8A** corresponding to the 2 obvious largest peaks at RT = 58.75 and 59.60 min (in **figure 4-7**). The absorption maxima of these spectra are consistent with those reported for lutein isomers. The details particularly the intensity ratios and wavelength intervals between the first and second main absorption peaks, and between them and the *cis* peak (denoted as DII, DIII and D_B respectively [*209*]) give useful information on identification of isomers. The small *cis* peak in spectrum E suggest that this is all-*E*(*trans*) lutein, while the hypsochromic shift in the peak maxima and moderate sized *cis* peak of spectrum D is consistent with it being a 9Z-*cis* isomer with the double bond more peripheral[*208, 210-212*].

The UV/Vis spectra of the peaks observed on the 440 nm chromatogram (**figure 4-2**), while clearly containing mixtures of fatty acid esters as shown by the mass chromatograms, nevertheless, showed a clear alternating pattern of all-*trans*-carotenoid/*cis*-carotenoid peaks, with the *cis* forms being present in larger abundance. Therefore, for every combination of 2 fatty acid chains (i.e. C18:0/C18:0, C18:0/C18:1, C18:0/C16:0, C18:0/C14:0, C18:0/C12:0, C18:1/C18:1, C18:1/C16:0, C18:1/C14:0, C18:1/C12:0, C16:0/C16:0, C16:0/C14:0, C16:0/C12:0, C14:0/C14:0, C14:0/C12:0 and C12:0/C12:0) there are 2 lutein forms (just considering peaks D and E). However with at least 6

other isomers identifiable from the spectra, this brings the possible number of di-ester forms up to at least 90. With fragmentation in MS this reduces to 30 combinations of mono-esters appearing during analysis, though regio-selective identification of isomers in respect to positioning of *cis* bonds relative to the fatty acid may be possible. With co-elution of these species and the greater relative abundance of C18:0 and C16:0 fatty acids the pattern observed in the UV-Vis chromatogram (figure 4-1) of approximately 14 peaks is explained. These peaks are labelled in **Figure 4-5** with identifications listed in **Table 4-3.**

The absorbance spectra of the other isomers (peaks A, B, C, D, E and H in **figure 4-7**) are shown in **Figure 4-8B.** The small *cis* peak and lack of hypsochromic shift is consistent with peak A being all-*E*-epi-lutein, while the l shifts and larger *cis* peaks of peaks D and E suggest 13-Z-isomers[*205, 212-215*]. The larger hypsochromic shifts exhibited by peaks A and B may be due to combinations of the 9 and 9'-*Z* or 13 and 13'-*Z* isomers (i.e. di-*cis* forms) and the relatively small *cis* peaks suggest the latter combination[*209, 212, 213*]. Peak H exhibits a hypsochromic shift similar to the assumed 13 –*Z* isomers but without the prominent *cis* peak so that a possible identification is problematic. The lack of a similar spectrum to that exhibited by peak F suggests that peak F is a merged combination of the 9 and 9'-*Z* isomers.

The saponified extract was found to contain 15.71 % free lutein isomers (based on the response of a lutein standard). Assuming complete hydrolysis and recovery of the lutein during the saponification process, this equated to a yield of free lutein from dried flowers of 0.37 % or approximately 0.7 % of lutein di-esters based on the relative areas of the peaks (and thus on the proportions of the fatty acids lengths) in **Figure 4-2**. The unsaponified extract would therefore have contained approximately 25 % lutein di-esters.

The pattern and quantity of the pigments present was not significantly different in the extract from the fresh (undried) flowers indicating that drying had not caused significant degradation or isomerization.

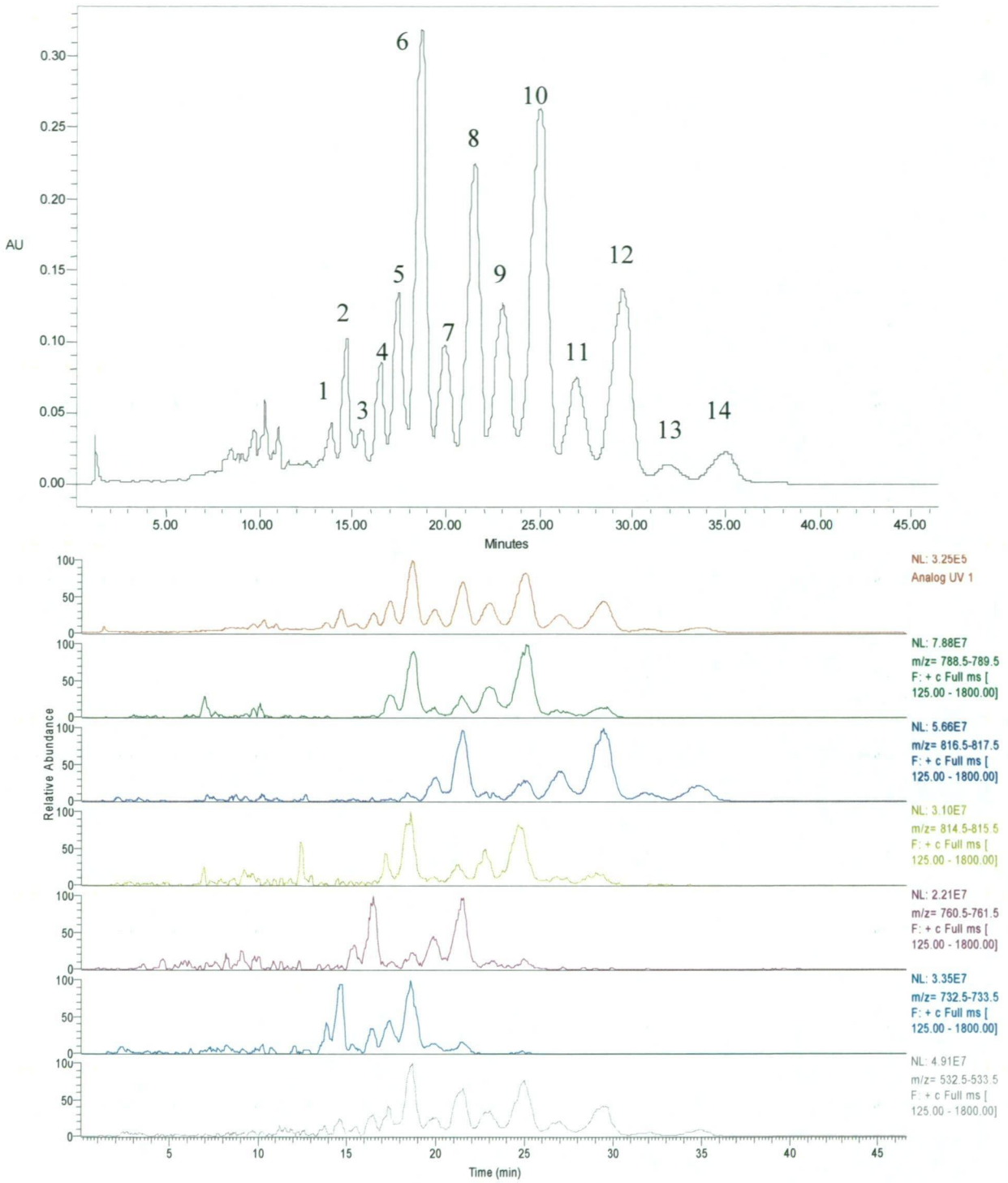


Figure 4-2: HPLC/UV and MS chromatograms of crude hexane extract of waste Crocus flowers (petals and stamens). Profiles include absorbance at 400 nm (top) and specific mass plots (below) showing the repeating pattern of 8 peaks evident for C16:0, C18:0, C18:1, C14:0 and C12:0 mono-esters and the protonated dehydrated free carotenoid.

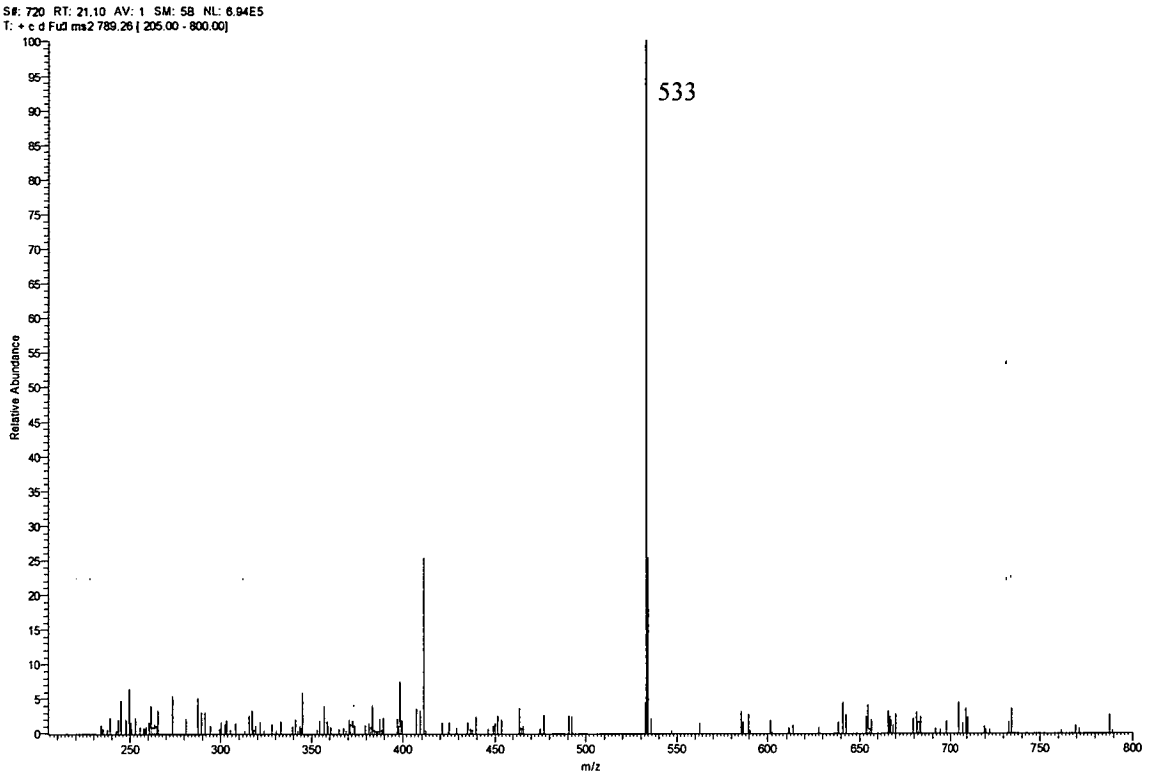


Figure 4-3: MS/MS daughters of m/z 789 eluting at 21.1 mins. (35% collision energy).

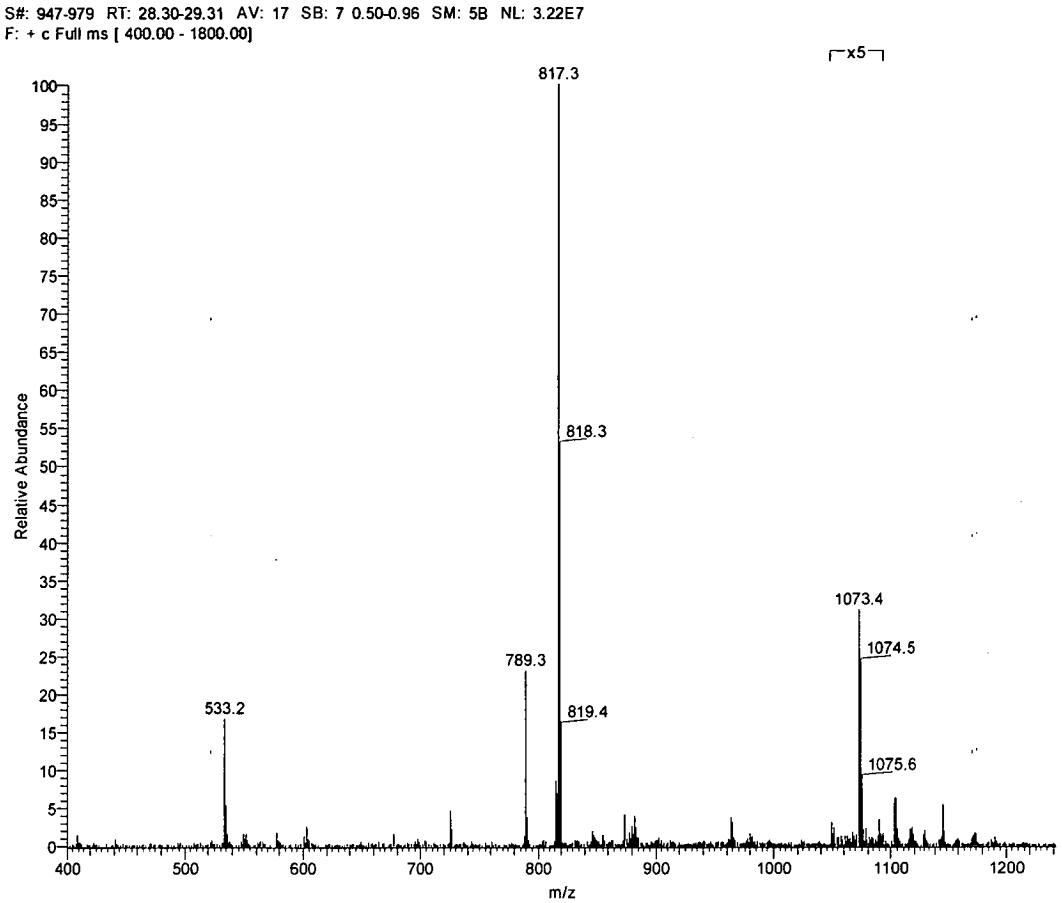


Figure 4-4: An example of mass spectrum of carotenoid ester peak (from RT = 29 min. in figure 3-2) showing trace detection of di-ester molecular ion (*m/z* 1074, magnified x 5), monoester fragment (*m/z* 818), free dehydrated parent molecule (*m/z* 533 = $[M+H-FA-FA]^+$) and the lack of a *m/z* 550 ion indicative of fragmentation of monoesters of lutein.

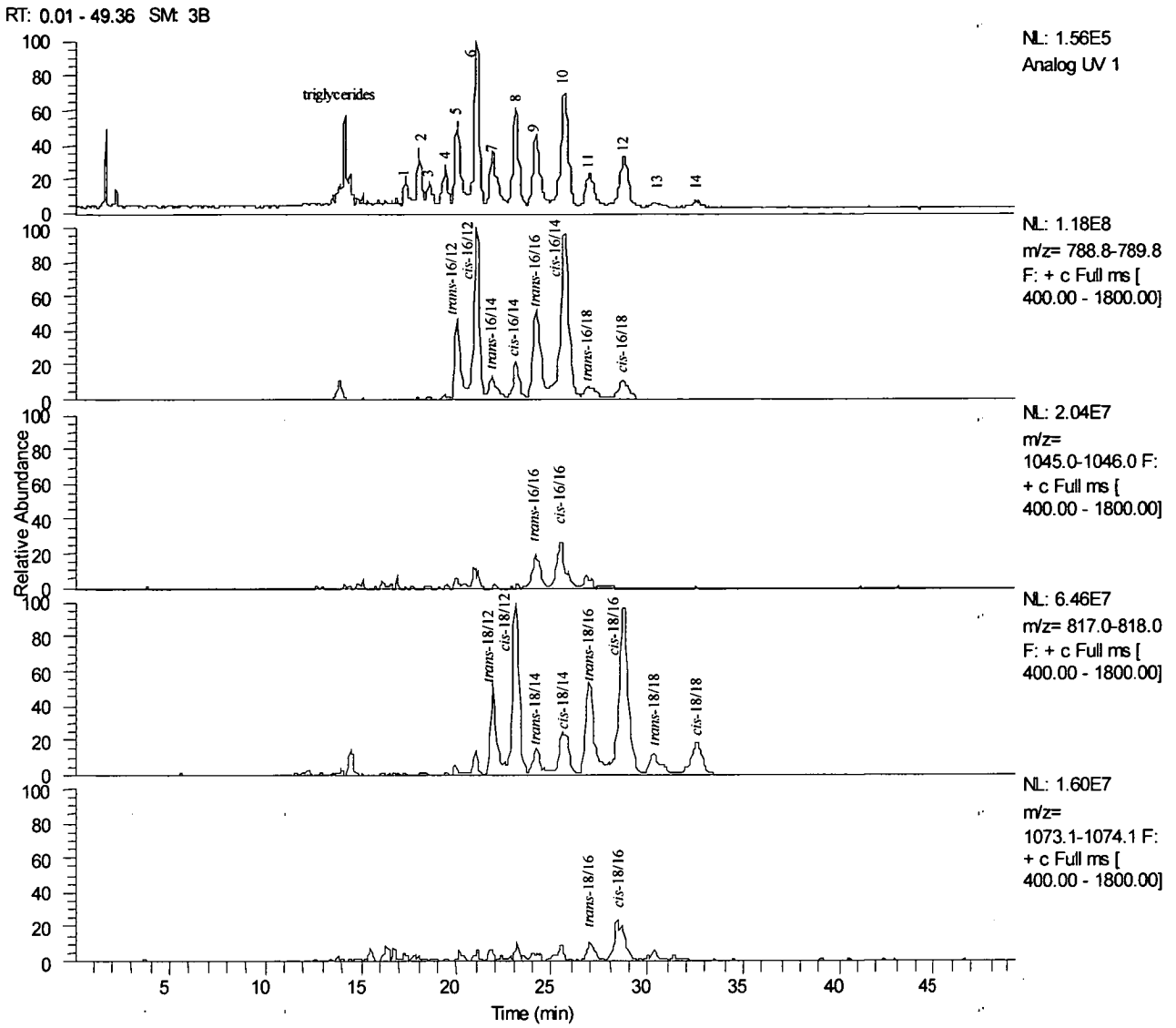


Figure 4-5: HPLC/UV and MS chromatograms of crude hexane extract of waste Crocus flowers (petals and stamens). Profile includes absorbance at 400 nm (top) and specific mass plots for 2 of the mono-ester fragments (C16:0 at m/z= 789 and C18:0 at m/z= 817) and the trace response for their di-ester parent molecules (assuming loss of a C16:0 fatty acid each). The identification of the peaks in the absorbance profile is given in Table 4-3.

Table 4-3: Peak identification for LC-UV/Vis absorbance profile given in Figures 4-2&4.

Peak number (see figure 4-4)	Identity, configuration and combinations of lutein di-ester carbon chain lengths
1	*trans* – 12/12
2	*cis* – 12/12
3	*trans* – 12/14
4	*cis* – 12/14
5	*trans* – 12/16 + 12/18:1 +14/14
6	*cis* – 12/16 +12/18:1 + 14/14
7	*trans* – 12/18 + 14/16 + 14/18:1
8	*cis* – 12/18 + 14/16 + 14/18:1
9	*trans* – 14/18 +16/16 + 16/18:1
10	*cis* – 14/18 + 16/16 +16/18:1
11	*trans* – 16/18 + 18/18:1
12	*cis* – 16/18 + 18/18:1
13	*trans* – 18/18
14	*cis* – 18/18

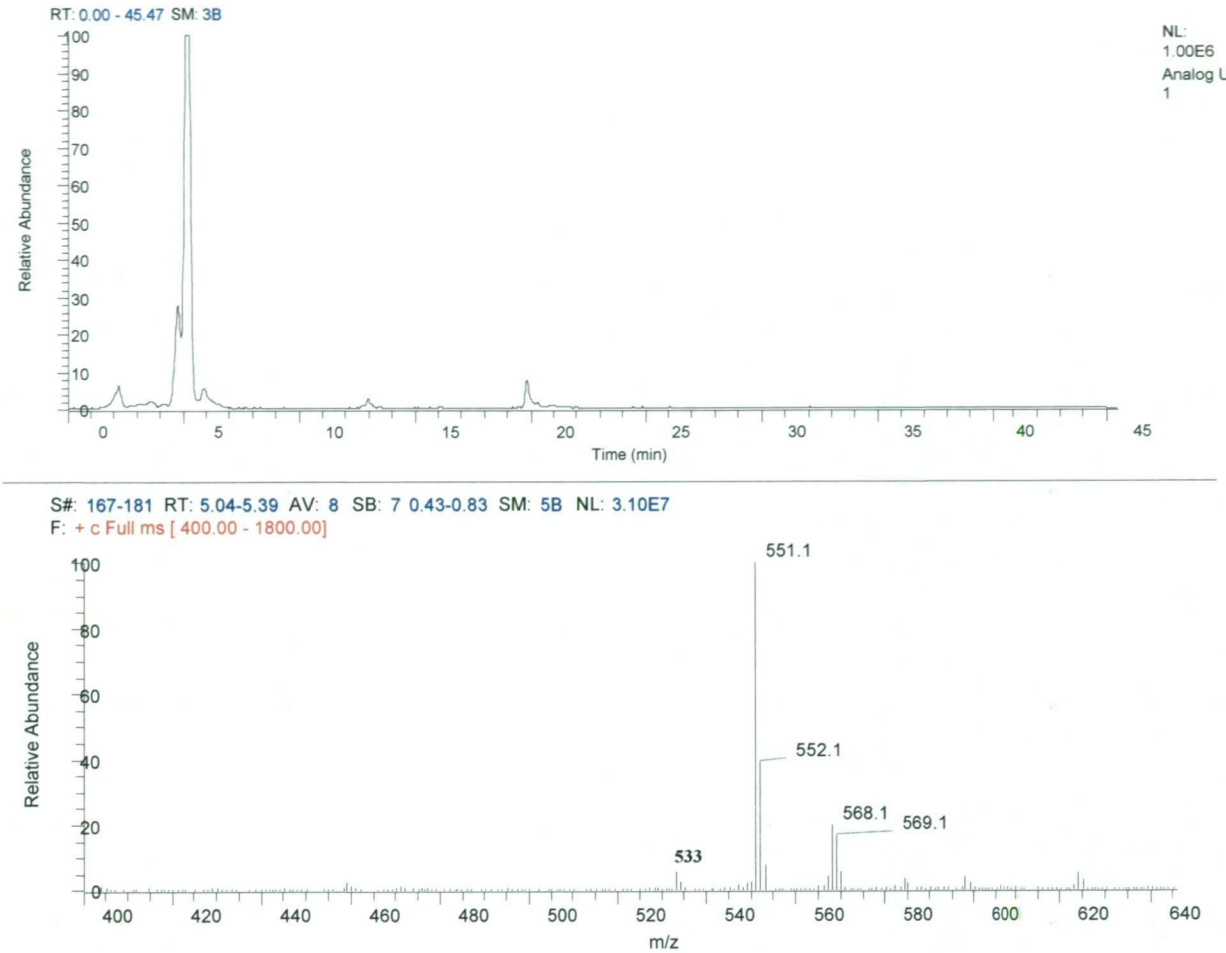


Figure 4-6: HPLC chromatogram (top) of saponified waste flower extract with MS/MS spectrum of the main peak (below).

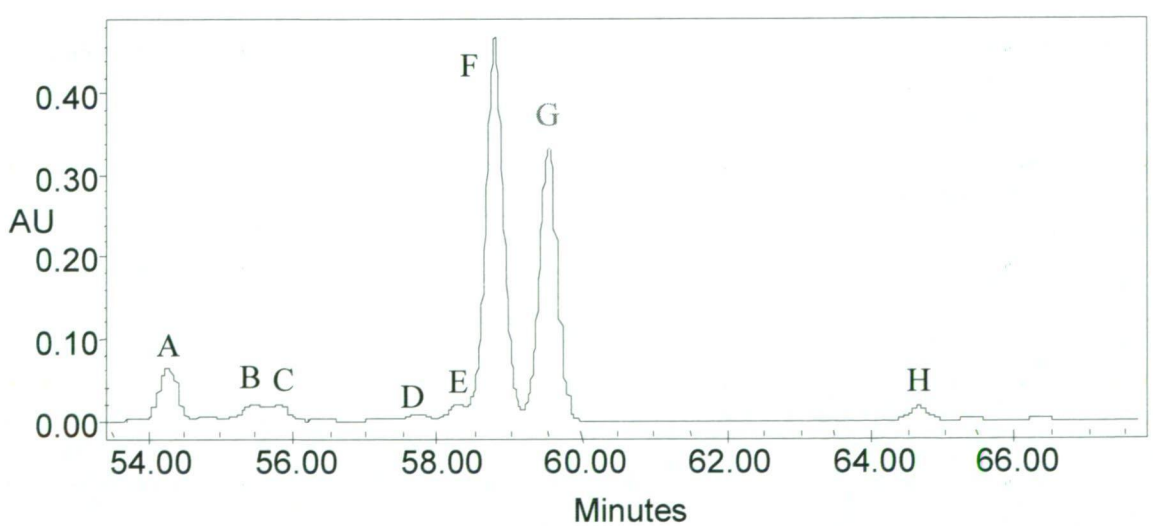


Figure 4-7: HPLC chromatogram of saponified waste flower extract run on polar solvent gradient showing improved peak separation of isomers with two C18 columns employed in series.

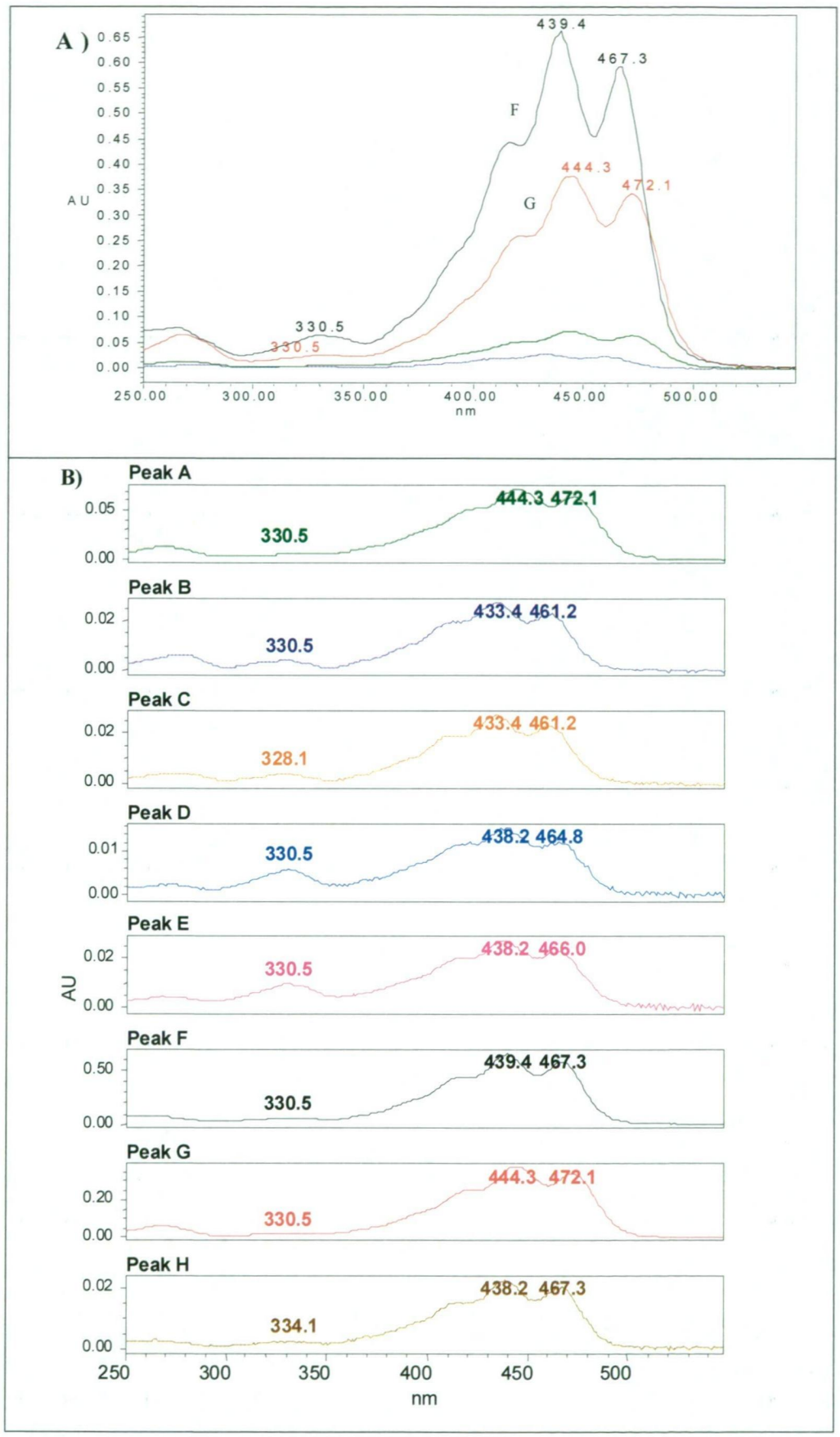


Figure 4-8: Absorbance spectra of A) the principle components (peaks F & G in fig. 4-7) of the saponified waste flower extract; and B) an expanded view showing isolated spectra of all peaks.

The GC-MS analysis of sample 4b (see **figure 4-9**) revealed that the extract contained only a relatively small proportion of aroma volatiles (the pre C18 peaks on chromatogram ≈ 1.3% of total area). Besides safranal and HCC (0.11% and 0.19% of total area), the extract contained identified volatiles including **1** = phenlyethyl alcohol & isophorone (0.08%), **2** = 2,6,6-trimethyl-2-cyclohexene-1,4-dione (0.01%), **3** = phenylacetic acid (0.09%), **4** = 4-hydroxy-3,5,5-trimethylcyclohex-2-enone(0.02%), **5** = unknown first reported as peak 29 by Kanakis *et al* [*68*](0.05%) and **6** = 5,1-hydroxyhexyl-tetrahydrofuran (0.13%). The bulk of the eluted peaks consisted of (in order of abundance) alkane and alkene waxes, long chain hydrocarbon alcohols and fatty acids (including **7** = palmitic (8%) and **8** = linoleic (3%) acids).

No conclusions may be drawn about the relative importance to the aroma of the extract as described in **Chapter 4.D** other than safranal is a major contributor. To gain such information GC-olfactory analysis would be required.

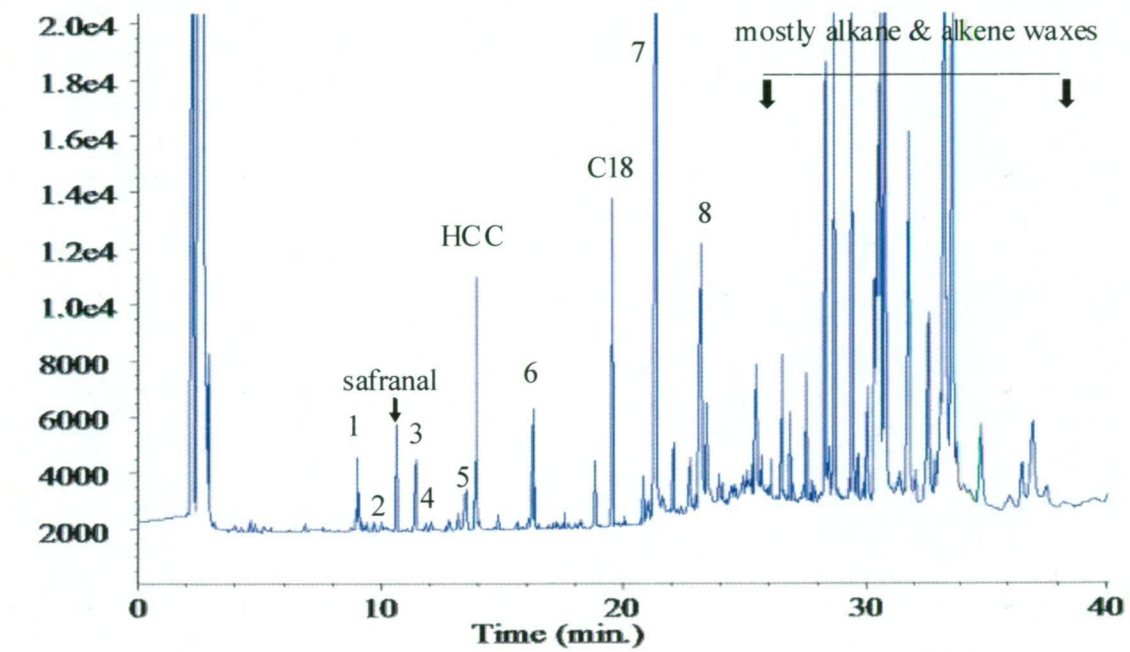


Figure 4-9: Gas Chromatogram of waste flower hexane extract (refer to text for peak identification derived by comparison to published data cited in chapter 2.B.6).

4.G Composition of semi-polar extract products

Introduction

Previous studies have described the extraction of flavonoids (specifically the kaempferol derivatives; kaempferol-3-sophoroside, kaempferol-3-sophoroside-7-glucoside and kaempferol-3,7,4'-triglucoside) from saffron spice[*30, 101, 216*]. Other reports indicate the extraction of kaempferol and quercetin glycosides from the tepals of *Crocus* flowers[*100, 217, 218*] but do not mention the stamens or give quantification. The purpose of this analysis was to confirm the presence of such compounds, further identify their conjugated structures and provide quantification to allow an estimate of the value of the waste flowers as a source of such potent bioactive compounds.

Experimental

Methanol extracts of fresh waste flowers were filtered (through Whatman #2 filter paper in a Buchner funnel) and analysed by LC-MS according to the conditions detailed in **Chapter 2.B**.

Results and discussion

The LC-UV chromatogram of the methanol extract is shown in **Figure 4-10**. The MS analysis of these peaks indicated that they are glycosides of 286 and 302 MW flavonoids. While a number of studies have reported the composition of kaempferol glycosides in saffron filaments[*100, 101, 216, 217*], a very recent study has now detailed the occurrence and abundance of kaempferol and quercetin glycosides in extracts of the tepals and stamens of *Crocus* flowers[*202*]. The inferred combinations of hexose units are shown in **Figure 4-10,** but comparison with known standards was required for actual identification in the absence of NMR data.

The results presented here (begun before the this recent publication) confirm the findings of Vignolini *et al* [*202*]. From the comparison of the mass spectra of these peaks with those of a standard mixture of flavonoid glycosides from *Brassica*[*169*], an example of which is shown in **Figure 4-11**, it is apparent that the predominant peak is kaempferol-3-sophoroside which is in agreement with the recent report. The other peaks, also identified by comparison to the *Brassica* extract are shown in **Figure 4-12** where single ion monitoring (SIM) was used to delineate the retention times of the different compounds. Their position on the HPLC- UV/Vis chromatogram is then indicated in **Figure 4-13**. Given that the characterisation of these flavonoids in saffron flower parts had already been achieved by Vignolini *et al,* neither the identification of the minor species, or the concentrations of compounds in separated stamens or tepals, were pursued.

Vignolini *et al* [*202*] found that the proportions of the various flavonoids did vary greatly between tepals and stamens, with the former richer in Kaempferol-sophoroside and the latter richer in many of the quercetin species. Quantification of the extract of the combined flower mass detailed gave the overall flavonoid content at 1.4% of fresh weight with the main component, Kaempferol-3-O-sophoroside present representing ≈ 90% of this yield. This is again consistent with the findings of Vignolini *et al.*

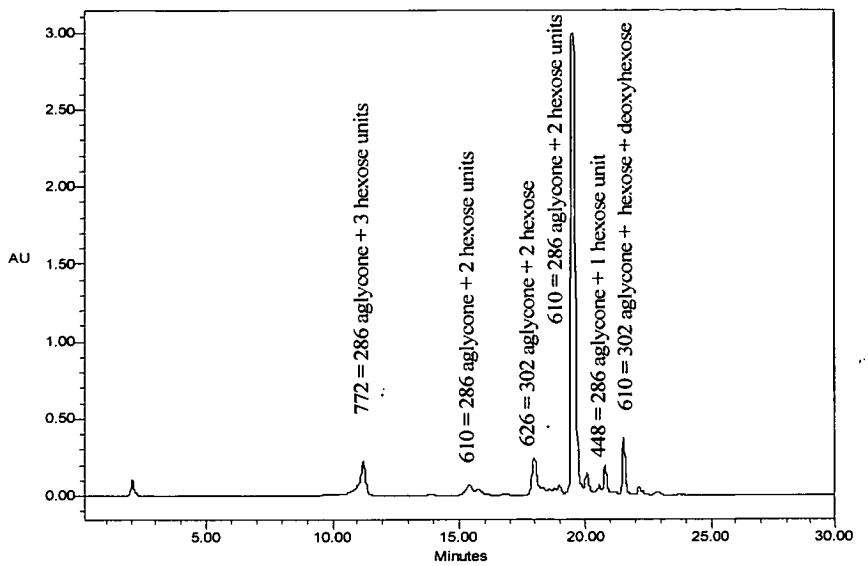


Figure 4-10: LC-UV chromatogram (at 330 nm) of methanolic extract of waste flowers with MW values (determined by LC-MS) and deduced flavonoid/hexose combinations annotated.

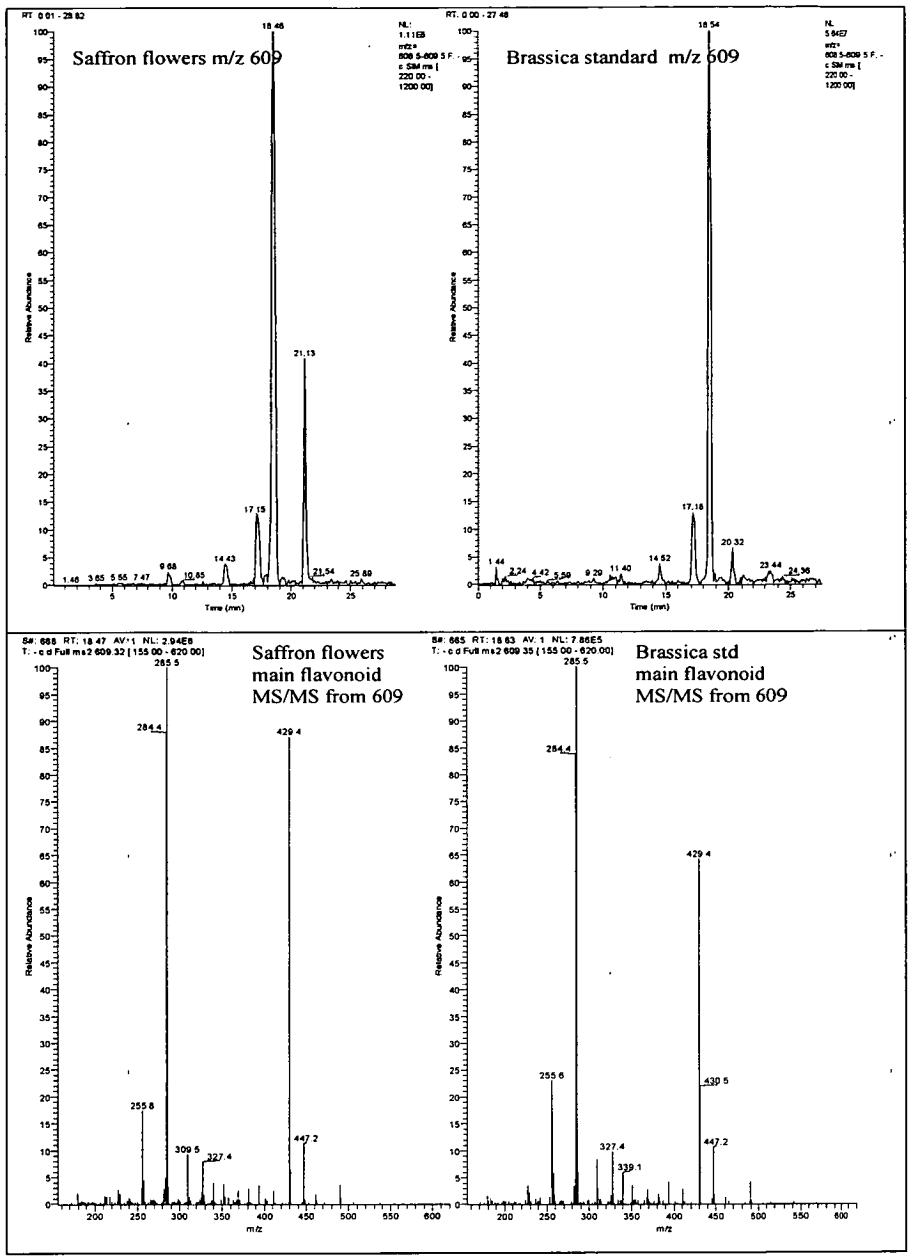


Figure 4-11: Comparative LC-MS profiles of saffron flower extract and Brassica standard in single ion monitoring mode at *m/z* 609 showing co-elution of peaks (top) and comparison of mass spectrum of kaempferol–3-O-sophoroside (molecular ion at m/z 609) confirming this identification in saffron extract

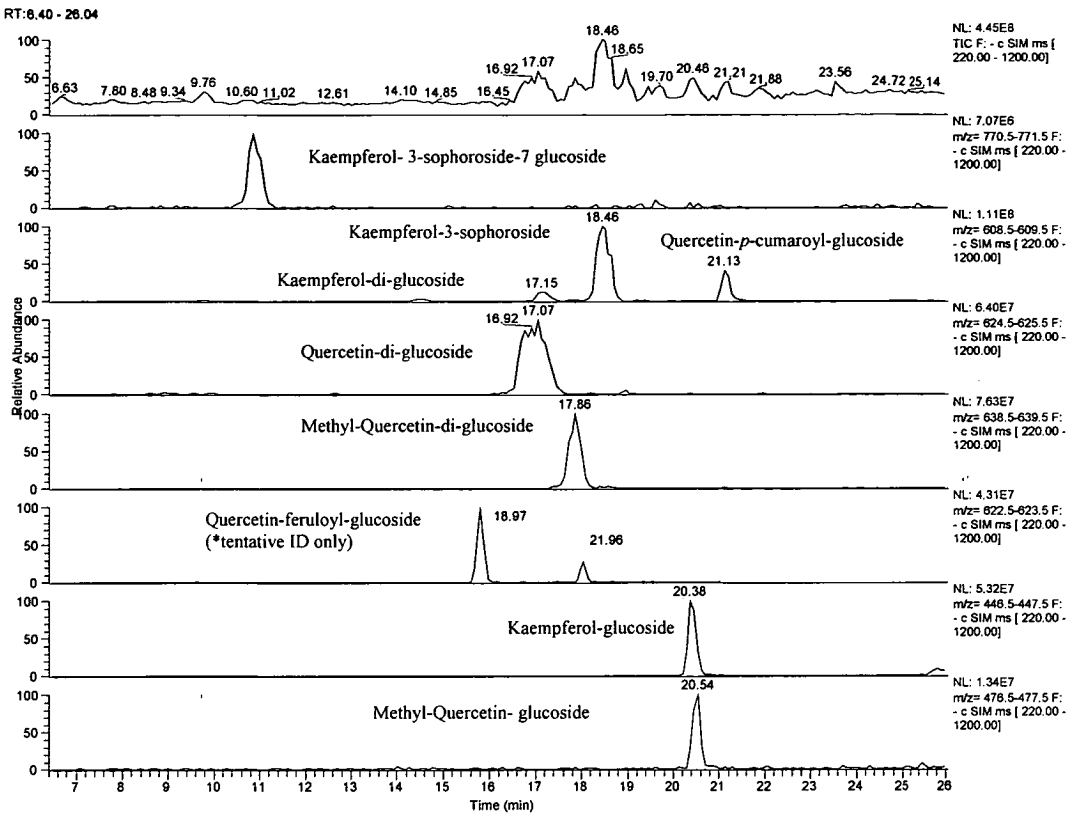


Figure 4-12: MS scan of saffron extract in SIM modes with identification of flavonoid glycosides at each MW.

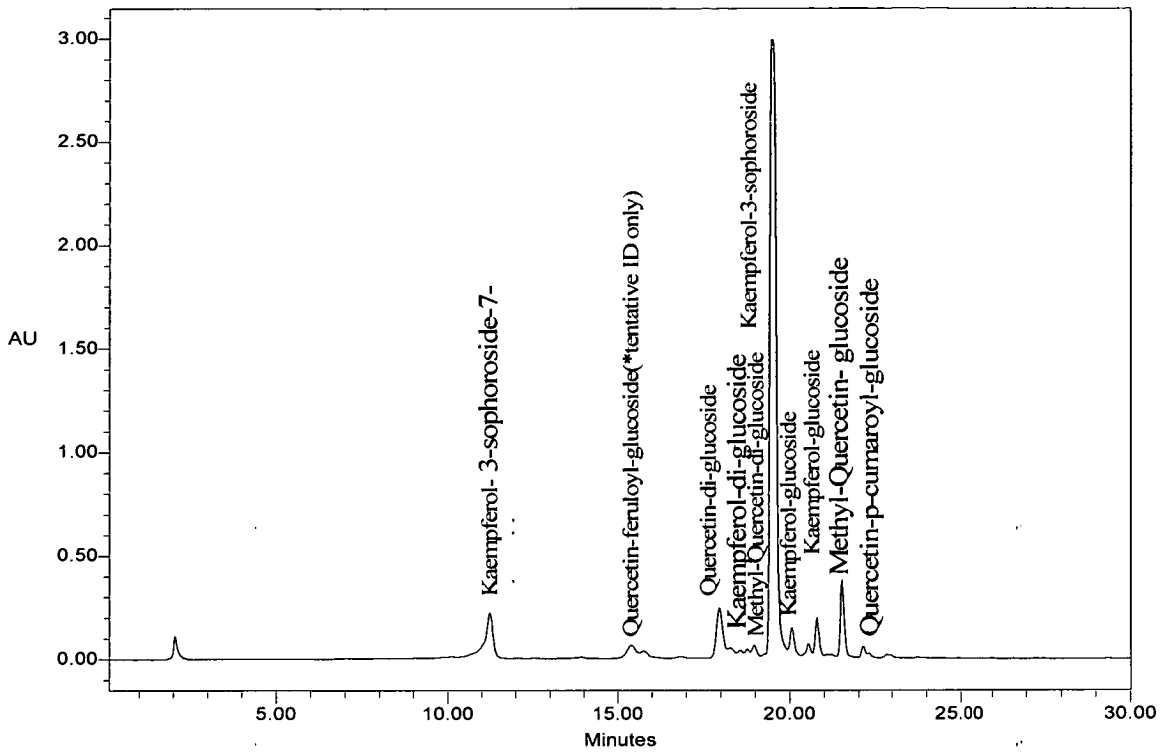


Figure 4-13: HPLC-UV/Vis chromatogram of waste flower methanol extract showing identification of flavonoid peaks.

4.H Discussion and Implications

The mean fresh flower weight for all the fresh flowers used in the extraction and distillation trials in this study was 0.99 g per flower. Based on the current local saffron production of approximately 3 kg per year[5] (where approximately 250 000 flowers are required to produce 1 kg of saffron), then the current annual production of waste flowers would be approximately 750 kg of fresh weight. Given average moisture content for these flowers of 0.86 % and an extraction yield (from dry weight) of 2.73%, the possible production of extract (concrete) per year would be approximately 3 kg.

This amount of extract may not be sufficient to create a viable market and would need to be priced higher than indicated in the Perfumer's comments to be worth pursuing as an option for by the Industry. However, there is a considerable stock (many 100g) of saffron product that is not of sufficient quality for sale due to degraded colour, but which has relatively high safranal content. The addition of appropriate quantities of this to waste flowers for extraction could result in product of considerably greater aroma strength that would likely attract a much higher price per kg. Clear identification and quantification of extract constituents with health care properties (alluded to in the perfumers comments) could also boost the price considerably. It should be remembered that safranal is one such compound.

Successful establishment of an extract as a commercial product could lead to a considerable increase in the volume of the *Crocus* crop with a proportion of it possibly exclusively grown for extract production. There is at present an excess of corm stock and thus a significant increase in production could be achieved relatively easily if the individual growers were given sufficient

incentive. If part of the crop were grown just for extract product then the considerable labour involved with the careful separation and drying of stigmas to produce saffron would be removed from the cost of this production as flowers could be picked and dried en masse at ambient temperature before storage and extraction. Use of the rejected saffron stock could be used to boost the aroma strength of the extract (during the initial years of market establishment), before crop production increased enabling the use of a proportion of the crop solely for extraction (and thus maintenance of consistently high safranal content).

Reports in the literature indicate that saffron flowers contain significant contents of potential aroma precursors such as glycosidicly bound carotenoid and non carotenoid metabolites[*9, 47, 166*] that are non-volatile and water soluble). Some form of fermentation treatment similar to those demonstrated in products such tea, tobacco, rose[*219*] and Boronia[*220*] could be used to cleave off the sugar units from these molecules, thus releasing aglycones as aroma volatiles that may improve the yield and aroma strength of extract products.

The contents of lutein di-esters and flavonoid glycosides (which compare favourably with other sources of these compounds) provide further potential for extracts having commercial value for their anti-oxidant health care properties. However, it may be very difficult to incorporate all of these attributes into the one product due to the very different polarities required to extract them.

5 GENERAL DISCUSSION

5.A Saffron Drying and Quality

5.A.1 Saffron quality measurement and the need for separate methods to quantify components of different polarity

While a number of authors have previously alluded to the inaccuracy of the ISO (aqueous extract) method for determination of saffron aroma strength (due to safranal content)[*28, 33, 68, 69*], there has been insufficient statistically significant evidence presented in the literature to prove this assertion. The comparative analysis presented in this work (see Chapter 3.A.2 and also **figure 3.6**) has now confirmed that the lack of solubility of safranal in water, as well as interfering absorption of *cis*-crocins, makes the ISO method inappropriate for assessment of safranal contents.

Despite this, it might be thought that with the use of a relatively polar solvent, a reasonably accurate measure of relative safranal content might be achievable (even if only a small proportion of the safranal was dissolved) and that – if not aqueous – a mid polarity extraction (eg ethanol, methanol or acetone) would allow this. Indeed (as some authors have attempted to demonstrate[*34, 69, 70, 87*]), an accurate determination of all the main components in the one extract would be desirable. However, the problem with polar extraction of safranal is not only one of solubility and interfering absorption (which is not an issue if chromatography is used). This is because polar solvents also extract picrocrocin which may subsequently hydrolyse to form safranal[*28, 33, 68, 69*] giving a higher level than in the original spice.

Furthermore, as Carmona *et al* [*32*] have alluded to, the effect of different drying methods on the micro-structural characteristics of saffron filaments is important. They suggest that different conditions may significantly affect the rate and completeness of crocin extraction; a proposition supported by the findings presented in chapter 3.G of this work. Significant differences in the access of polar solvents to safranal are caused by different drying conditions affecting the inter- and intra-cellular structure of stigma tissues. Such differences may still be apparent even when samples are ground before extraction because cells (and cell membranes) may remain physically intact, and so the relative resistance of the membranes to solvent/solute movement affects safranal extraction. The conditions of drying, particularly temperature and humidity are critical to the integrity of such membrane barriers.

For all of these reasons it is recommended that, for valid determination of the true contents of safranal, picrocrocin and crocins in saffron, 2 separate extractions (polar for picrocrocin and crocins and non-polar for safranal) are required.

5.A.2 Australian saffron quality and the effect of degree of drying and storage on colour and aroma

The results of the survey of saffron samples supplied by Australian Growers between 2004 and 2007 presented in Chapter 3.A.3, indicated that, while the local product generally rated very well for colour strength compared to imported products, considerable potential for enhancement of aroma strength of the local product exists. Furthermore, the use of a low temperature drying method (to produce the local product) was shown to present considerable risk of under-drying of filaments resulting in unwanted degradation of pigments (crocins) during storage (see chapter 3.A.4). While such "damp" storage may also result in increased safranal content due to post drying enzymatic hydrolysis, it can cause generation of unwanted compounds giving off-notes to the product[*50, 51*].

The storage trials (see chapter 3.A.4) also showed that if saffron is sufficiently dried, it may be stored for at least 2 years without significant degradation (given the correct storage conditions of low humidity and light). This challenges the assertions of Riana *et al*[45] and Ordoudi and Tsimidou[*10*] that storage should be limited to < 1 year to preclude quality deterioration.

The existence of saffron product with high contents of both pigments and safranal, such as the Spanish samples analysed in Table 3.3 and reported by Alonso *et al*[*33*]indicates significant potential for improvement of local product by enhancing aroma development without excessive pigment degradation. It is difficult to see how this might be produced by "soft" (enzymatic) hydrolysis – during low temperature drying or in subsequent storage – without some form of higher temperature blanching period to deactivate browning enzymes (polyphenoloxidases). However, this would also deactivate β-glucosidase and thus prevent enzymatic production of safranal. Of all the samples analysed during the storage trial (see Chapter 3.A.4), those with safranal contents nearing that of the best Spanish samples had much reduced colouring strengths. Thus, some other method of drying (other than low temperature) must be required for both optimal colour retention and aroma development.

5.A.3 The benefits and side effects of high temperature drying.

The traditional use of drying methods incorporating the use of charcoal embers or other radiating heat sources in Spain did suggest that high temperature drying might be responsible for the best quality products despite a long held belief that the temperatures of the saffron filaments reached during such methods were relatively mild[*28, 32*]. More recently the temperatures during such drying have been quantified and are reported in the range 75-121 °C[*32*].

This and the work of Loskutov *et al*[*38*] who found more safranal produced by drying at 80 °C (a temperature that would likely result in deactivation of pigment degrading enzymes), provided good reason to investigate the potential for drying saffron at elevated temperatures despite the findings of Riana *et al*[45] and Pardo *et al* [*69*], and the conclusions expressed in the reviews of Cadwallader, Fernandez and Tsimidou[*6, 9, 10*], that temperatures between approximately 35-45 °C are optimal for saffron drying.

The results detailed in Chapters 3.B and 3.C of this work show this to be the case as there was significantly more safranal produced in saffron by temperatures between 80-90 °C. The use of such temperatures also reduced the loss of crocin pigments and/or increased extractability of these compounds. These finding are at odds with the previous conclusions of Cadwallader, Fernandez and Ordoudi and Tsimidou[*6, 9, 10*] regarding optimum saffron drying conditions but are consistent with the more recent propositions of Carmona *et al* (06, 07). Moreover, they have hypothesised that high temperature drying not only results in saffron with higher safranal content, but may also increase the content of precursor compounds (other than HCC) from crocin degradation, which may then be converted to safranal during cooking (i.e. at temperatures > 100 °C).

The pigment degradation prevention effect is due to either more rapid drying acting to reduce water activity below that required for polyphenoloxidase activity and/or through direct thermal deactivation of such enzymes. The rehydration and β-glucosidase inhibitor experiments (in chapter 3.H) showed that the increased safranal production from high temperature drying occurs primarily via direct hydrolysis and not from enzymatic conversion of picrocrocin during a temperature lag phase induced by evaporative cooling (see chapter 3.D). However, even with increased safranal content induced by high temperature drying, the bulk of the picrocrocin in the saffron remains un-hydrolysed and thus the potential for much greater safranal production remains.

The cause of the higher colouring strength/crocins contents of saffron dried at elevated temperatures also involves a component of greater pigment extractability. Changes in the cellular and sub-cellular structure of the filaments due to more rapid water loss and tissue shrinkage were correlated with more rapid and complete pigment diffusion in Chapter 3.G. A side effect of such changes is that filaments also become more brittle and thus less easy to package and sell as whole saffron threads (the desired form for marketing of premium product[*5*]).

Changes in crocin extraction rates do not however, account for the differences between various drying treatments observed in experiments detailed in chapters 3.B, C and D; both because the magnitude of the differences were greater than between treatments in chapter 3.G and because the extraction time for crocin determination (16 hours) was much longer (than in 3.G) to allow complete dissolution.

5.A.4 The negative effect of airflow during drying

The use of drying methods incorporating fans (and thus airflow) occurs commercially (including in the Australian Industry and has been claimed to enhance saffron quality when dried at low-moderate temperatures[*9*] and at elevated temperatures[*69*]. The effect of airflow is to speed drying and therefore limit the time when pigment degradation can occur, resulting in enhanced pigment retention and thus colouring strength. Conversely however, the findings detailed in Chapter 3.C.4 clearly show that airflow (at both high and low temperatures) impacts negatively on safranal content of the resulting saffron, without improved retention of crocins. Indeed, under some conditions, airflow may also have a negative effect on pigment retention; possibly due to evaporative cooling delaying thermal inactivation of polyphenoloxidases by lowering humidity around the stigmas (see figures 3.8, 3.9 and 3.15).

The reduced safranal from use of airflow may be (in part) due to more rapid drying limiting the period when conversion of picrocrocin may occur. Nevertheless, the measurement of safranal in the exhaust from the drying treatments suggests that (particularly at the higher temperatures) loss of safranal from the filaments by evaporation is a significant factor.

5.A.5 The importance of water activity (humidity) during high temperature drying.

None of the previously published comparative studies of different saffron drying conditions have described or even referred to any evaporative cooling effect in the stigma tissue[*28, 32, 38, 45, 68, 69*]. Presumably the temperatures quoted in these reports refer to the temperature applied (thermostat reading of the heating device or measurement of air surrounding the stigmas). This study has shown that significant evaporative cooling does occur, and is particularly marked when elevated temperature drying (90 °C) is applied (see Chapter 3.D.2). While this raised the possibility that much of the increased safranal production could be due to rapid enzymatic activity made possible by the suppression of temperature to below that that where β-glucosidase is inactivated, the experiments in Chapter 3.H showed that high temperature conversion occurred despite previous thermal inactivation or chemical inhibition of the enzyme and was thus independent of evaporative cooling.

The comparative drying treatments applied (at variable humidities) in Chapter 3.D.1 and the characterisation of such drying runs regarding humidity and stigma temperature measurement (see Chapter 3.D.2) show that humidity is critical to the production of safranal by direct conversion at higher temperatures. As correctly suggested by Tsimidou and Biliarderis[*35*], the release of volatile aglycones from (non enzymatic) hydrolysis of terpenoid glycosides (such as picrocrocin) is favoured by intermediate water activities. During saffron drying, this range (0.43- 0.53 a_w) equates to approximately half dry stigmas – and so by slowing the rate of water loss during high temperature drying, the stigmas are kept within this range for much longer allowing significantly more safranal production. While complete conversion of picrocrocin was not achieved by this method, the gain in safranal was at least twice that from any other method or experiment reported in the literature[*30, 33, 34, 37, 38, 45, 46, 49, 50, 68, 70, 87, 221*].

This slowing of water loss from stigmas; achieved by elevating humidity, also acts to reduce the evaporative cooling effect. Therefore during drying, the (actual) temperature of the stigmas rises to above 70°C more rapidly. This has two effects: that a higher rate of hydrolysis of picrocrocin (to form safranal) and the "blanching" effect (of polyphenoloxidase deactivation) are both achieved earlier in the drying period.

The effect of elevated humidity also acts to reduce the intercellular tissue disruption by slowing tissue shrinkage[*32*]. Such shrinkage leads to excessive filament brittleness, but by slowing drying the filaments may remain more structurally resilient. What high humidity drying does not do is reduce membrane disruption as drying temperatures are not reduced (and in fact are higher for some time during drying due to reduced evaporative cooling). This allows pigments to be more rapidly and completely extracted than drying at low-moderate temperatures, effectively increasing colouring strength of the product, as was demonstrated in Chapter 3.G.

The benefits of the high temperature/humidity method were demonstrated on a commercial scale in Chapter 3.F. In retrospect, it now seems likely that the Spanish method of toasting stigmas over hot coals can produce high quality saffron partly because the mass of stigmas is large and dense enough to create an extended period of elevated humidity in the middle of the mass were the high temperature/humidity method effectively occurs.

5.A.6 The chemical pathway of safranal production at elevated temperature and humidity.

As outlined above, the experiments in Chapter 3.H have established that the higher safranal production achieved at elevated temperatures may be attributed to direct (non-enzymatic) hydrolysis. The proposed alternative pathways (involving thermal degradation of crocins - see Chapter 1.B.2) proposed by Carmona *et al[28, 29, 37]*, were prompted by inconsistencies observed [*38, 81*] from thermal treatments. These comprised picrocrocin disappearance without any accumulation of safranal; and further safranal accumulation in saffron initially devoid of picrocrocin. They also questioned why, given the large pool of picrocrocin usually remaining in saffron after drying, more safranal could not be generated by any of the many drying conditions tested. In effect, even in saffron with very high safranal levels (> 4000 ppm) this level represents < 10% conversion from picrocrocin (molar basis).

The analytical evidence (including identification of the possible intermediate compounds) from the experiments of Carmona *et al*[*37*] is very convincing in respect to this alternative pathway. However, although they were only able to demonstrate safranal generation from crocins (or crocetin) at temperatures >> 100 °C, their description of the process as "thermally accelerated storage" implies that they consider that this pathway occurs more gradually at much lower temperatures. Whether they propose that this alternative pathway is a source of safranal during initial drying of stigmas (as opposed to subsequent storage or thermal treatments) is unclear.

What is now clear from the hypothesis of Carmona *et al*[*29, 37*] is that they also consider drying at higher temperatures to be preferable in that more safranal precursors are thought to be formed during initial drying (at 80-90 °C), and these may then be subsequently converted to safranal upon cooking (> 100 °C). From the findings of this study there does not seem to be a need to go beyond the accepted pathway of safranal production (from picrocrocin) to explain the levels generated or the disappearance of picrocrocin during drying at ≈ 90 °C. The alterative pathway (from crocins) at >> 100 °C would not be practical for the commercial production of saffron. Such temperatures cause unwanted changes in the composition of the pigments; specifically via conversion of *trans* crocins to *cis* forms as shown by Carmona *et al*[32] (as well as the possible alternative safranal production pathway). This is readily observed as an obvious browning and dulling of the colour and the spice also becomes more brittle than is desired.

It may be concluded that this proposed alternative safranal generation mechanism should be considered an primarily a factor in saffron use (i.e. cooking) rather than saffron production (by drying), Nevertheless high temperature drying may maximize formation of precursors available for conversion to safranal upon cooking.

The experiments detailed in chapter 3.H also allow an assessment of the optimal post harvest treatment of stigmas prior to final drying. There have been suggestions[*9, 10, 35, 39, 45, 50, 68*] that by drying stigmas slowly (in effect incubating) at moderate temperatures (<< 70 °C), a large of pool of HCC is formed which may then be dehydrated to produce a high safranal content by a "burst" of temperature > 80 °C. This temperature also provides the "blanching" effect to prevent further pigment loss. The findings of the rehydration and β-glucosidase addition experiments (Chapters 3.H.1&2) show this not be the case. Although a large pool of HCC may be formed from enzyme activity this way, at elevated temperature (≈ 90 °C) it appears to be largely dehydrated to

compounds other than safranal, and some of these may actually detract from the aroma quality of the spice.

Chapter 3.H showed that it is optimal to minimise enzyme (β-glucosidase) activity in stigmas prior to drying by the high temperature/humidity method to maximise the pool of picrocrocin available for direct conversion. Stigmas should thus be dried as soon as possible after harvest. Similarly, as the harvest timing experiment (Chapter 3.E) indicated, the harvest of flowers in the early part of the day and just prior to petal opening, maximises the pool of picrocrocin available for direct thermal hydrolysis. It also minimises loss of colour due to photo-degradation.

The re-hydration experiments also indicate that it is possible to enhance the level of safranal in previously dried saffron. By re-hydration of filaments to achieve sufficient water activity, direct hydrolysis may then occur when filaments are re-dried at high temperature/humidity. Some pigment loss may occur during the re-hydration, though prompt application of the drying conditions would minimise this.

5.B Waste Flower Products

5.B.1 Distilled product

The distilled product, while containing compounds with flavour and fragrance properties, is not available at a yield sufficient to make it commercially interesting particularly as the product was noted to contain significant amounts of light waxes. These would need to be separated from the product as they mar the overall fragrance.

5.B.2 Non-polar extract product.

The most attractive flavour and fragrance product is obtained from the air-dried waste flowers by extraction with a non-polar solvent (hexane). To have sufficient aroma strength for commercial use the waste flowers must contain approximately 1% (w/w) of rejected stigma material as a source of safranal.

This extract was described by a leading Perfumist who indicated that it has potential as a commercial product.

Drying of the waste flowers by individual growers (necessary because of the logistics and costs of collection from widely distributed sources) was shown to be feasible by simple air-drying in a darkened shed.

The volume of waste flowers available at present, given the size of the Industry in Australia, is not sufficient to supply a market and provide a commercially viable return for the cost of production. Viability would require a major increase in economy of scale, possibly through larger areas of crop with incorporation of mechanical harvesting.

The extract consists of a small proportion of volatiles (<10%), waxes and carotenoid pigments which give the product a bright yellow/orange colour.

The pigments have been shown to be fatty acid di-esters (of various chain length combinations)) carotenoids. MS and absorbance spectra have allowed the putative identification of the carotenoids as isomers of lutein. These compounds have been commonly reported in the extracts of flowers and have significant anti-oxidant properties. However, it is unusual that the flowers contained only di-esters as previous reports of plant sources high in lutein esters report a mixture of mono- and di-esters.

5.B.3 Polar extract products

Polar (methanol) extracts of waste flowers were shown to contain significant amounts of flavonoids including quercetin and kaempferol glycosides. The latter have been positively identified as combinations of kaempferol sophorosides and glucosides, confirming the recent work by others. These compounds have been reported to have significant anti-oxidant properties linked to potential chemo-preventative uses, particularly in regard to cancer.

Comparison (UV and MS) with known standards has allowed confirmation of the identities of most of these compounds with the combined concentration of up to 10 % of dry wt of the flower parts. There are significant differences in the distribution of the flavonoids between stamens and tepals indicating that specific compounds could be targeted if a commercial use is found.

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