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Investigation of flavour compounds from sweet, semi-bitter and bitter almond kernels

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Abstract. A chemical basis for distinguishing bitter phenotype and the non-bitter phenotype from the semi-bitter one has been determined. In this study, kernels from 101 trees, all derived from a common maternal parent, were evaluated using three approaches: (i) sensory analysis; (ii) amygdalin quantitation using HPLC; and (iii) non-targeted analysis of volatile metabolites released on maceration by SPME, GC-MS, and head space MS electronic nose (MS e_nose). Tasting identified three categories of flavour (sweet, semi-bitter, and bitter) that were also distinguishable on the basis of chemical analyses and by MS e_nose using principal component analysis. Highly significant correlations were found between amygdalin content and the taste panel's score for "marzipan", and also between the taste panel's scores for sweet and overall taste preference. Non-targeted metabolite analysis identified benzyl alcohol and isomers of 2,3-butanediol as potentially important flavour components of almond for the first time.

Keywords. Rosaceae – *Prunus dulcis* – Mass spectrometry – Electronic nose – Chemometrics.

Recherche sur les composés du goût et de l'arôme pour des amandes douces, semi-amères et amères

Résumé. Une base chimique a été déterminée pour distinguer le phénotype amer et le phénotype doux par rapport au semi-amer. Dans cette étude, les amandes de 101 arbres, tous provenant d'un parent maternel commun, ont été évaluées en utilisant trois approches : (i) analyse sensorielle ; (ii) teneur en amygdaline en utilisant la méthode HPLC ; et (iii) analyse non ciblée des métabolites volatils libérés sur la macération par SPME, GC-MS, et nez électronique par spectrométrie de masse à espace de tête (MS e_nose). L'échantillon a identifié trois catégories pour la saveur (doux, semi-amer, et amer) que l'on pouvait également distinguer sur la base des analyses chimiques et par MS e_nose en utilisant l'analyse en composantes principales. Des corrélations fortement significatives ont été trouvées entre la teneur en amygdaline et la note attribuée par l'équipe de dégustateurs pour le "masepain", et également entre la note de cette équipe pour le goût sucré et la préférence globale. L'analyse non ciblée de métabolites a identifié l'alcool de benzyle et des isomères de 2,3-butanediol en tant que composants potentiellement importants de saveur d'amande pour la première fois.

Mots-clés. Rosaceae – *Prunus dulcis* – Spectrométrie de masse – nez électronique – Chimiométrie.

I – Introduction

Almond flavour is an important aspect in the manufacturing of almond products, whether it is for the fresh (raw) market or for processing into confectionaries such as nougat, "turrón" or candies. Almond oil is also extracted for food flavourings and the cosmetics industry. The enzymatic breakdown of cyanogenic glucosides in almond kernels and the concomitant liberation of hydrogen cyanide and benzaldehyde are responsible for the bitter taste of some kernels. Two related cyanogenic glucosides found in almond plants are the monoglucoside, prunasin, present in vegetative organs, and the diglucoside, amygdalin, that occurs only in the kernel (Frehner *et al.*, 1990). Bitter almonds contain 3-5% amygdalin, taste very bitter, and develop a characteristic cyanide aroma with moisture. Sweet almonds have a slightly nutty fragrance and taste, while semi-bitter almonds have a "marzipan-like" taste. However, a chemical basis to distinguish this

phenotype and the sweet phenotype from the bitter flavour has not been determined. In previous work by Dicenta *et al.* (2002), bitter kernels could easily be distinguished from sweet and semi-bitter kernels on the basis of their amygdalin content, but semi-bitter and sweet kernels were indistinguishable.

Here we extend that report by characterising a different set of genotypes and, in addition, analysing the volatiles released from ground kernels. Kernels from the sweet, semi-bitter, and bitter progeny of open-pollinated 'Mission' almond were thus differentiated by chemical analyses.

II – Materials and methods

1. Almond samples

Plant material was derived from an open-pollinated cross of the cultivar 'Mission'; semi-bitter (heterozygous; Ss). One hundred-and-one half-sib progeny were produced from this cross in 1998, as part of the Australian almond breeding program (Wirthensohn and Sedgley, 2002).

2. Kernel evaluation

Up to thirty fruit were picked from each progeny tree in March 2004. Kernels were classified as belonging to one of three flavour categories: sweet, semi-bitter, and bitter. Sensory evaluation was then undertaken by a panel of ten tasters to verify the initial categorisation of the almond kernels and to determine taste preferences for the different kernel flavours.

3. Measurement of the amygdalin content of kernels

High Performance Liquid Chromatography (HPLC) analysis was used to detect and measure the levels of amygdalin and/or prunasin in almond kernels. Including 'Mission', 78 genotypes were analysed (26 sweet; 38 semi-bitter; 14 bitter) with ten to 50 replicates of each. HPLC procedures follows that of Wirthensohn *et al.* (2008). Tests for correlation between the HPLC results and sensory evaluation scores were computed using Spearman's Rank correlation coefficient.

4. MS-Electronic nose

A Chemical Sensor (HP 4440) was used to evaluate the volatiles released from the macerated kernels. The instrument consisted of a modified HP 7694 automated headspace sampler, a mass sensor (based on an HP 5973 mass-spectrometry detector) and a data-processing module using Pirouette software. Natural groupings of almonds were analysed by principal components analysis (PCA) of the volatile compounds in the headspace of sweet, semi-bitter or bitter kernels. Including 'Mission', 51 genotypes were analysed (ten replicates each).

5. GC-MS analysis

One representative from each phenotype was analysed by GC-MS. Blank samples were run as controls. Almond kernels were ground separately using a mortar and pestle. Sample (1 g) was placed into a 15 ml headspace glass vial, 1 ml 40 mM 2-(N-morpholino) ethanesulfonic acid was added and the vial was immediately capped with a vial crimp seal fitted with a Teflon-coated septum to avoid any loss of volatiles. Four-point calibration curves were generated for benzaldehyde, benzyl alcohol and 2,3-butanediol. SPME fibre was exposed to the headspace above the sample in the glass vial. In all cases, the SPME extraction was carried out for 10 min at 35°C with agitation at 250 rpm. Analysis was carried out according to Wirthensohn *et al.* (2008).

III – Results and discussion

1. Kernel evaluation

Of the 101 genotypes evaluated, 14 bitter, 37 semi-bitter and 50 non-bitter trees were identified. The panelists stated their preference towards the sweeter and slightly semi-bitter almond flavours. This information helps the breeding program by directing crosses towards such flavour outcomes. Highly significant positive correlations existed between amygdalin content and the panel score for "marzipan" flavour (0.56), and between the panel score for sweet and their overall taste preference for the kernels (0.27), ($P \leq 0.001$). Positive correlations existed for overall taste preference and amygdalin content (0.22, $P \leq 0.01$) and between overall taste preference and "marzipan" flavour (0.20, $P < 0.05$). A significant negative correlation existed between the panel score for sweet, and the score for "marzipan" (-0.26, $P \leq 0.01$). This result directly links amygdalin content with "marzipan" flavour of semi-bitter almonds.

2. Measurement of the amygdalin content of kernels

HPLC analysis established the presence and level of amygdalin in almond kernels. The mean amygdalin levels found in bitter kernels was more than 500-times greater than the mean value for non-bitter kernels, and 90-times higher than the mean value in semi-bitter kernels. There was five-times the amount of amygdalin on average in semi-bitter kernels than in sweet kernels, where the amount of amygdalin was very low or absent. The level of amygdalin in 'Mission' was low, falling between sweet and semi-bitter levels (Fig. 1.) The range in amygdalin content between bitter kernels and the other categories did not overlap which would suggest a major gene effect, as suggested by Dicenta *et al.* (2002). Evidently manifestation of the sweet and semi-bitter flavours are more likely to result from reduced amygdalin accumulation in the kernel, rather than reduced amygdalin degradation (to benzaldehyde and cyanide). The amygdalin content of some sweet kernels are higher than some semi-bitter kernels, so it seems that amygdalin content may not be the only compound defining the difference between sweet and semi-bitter.

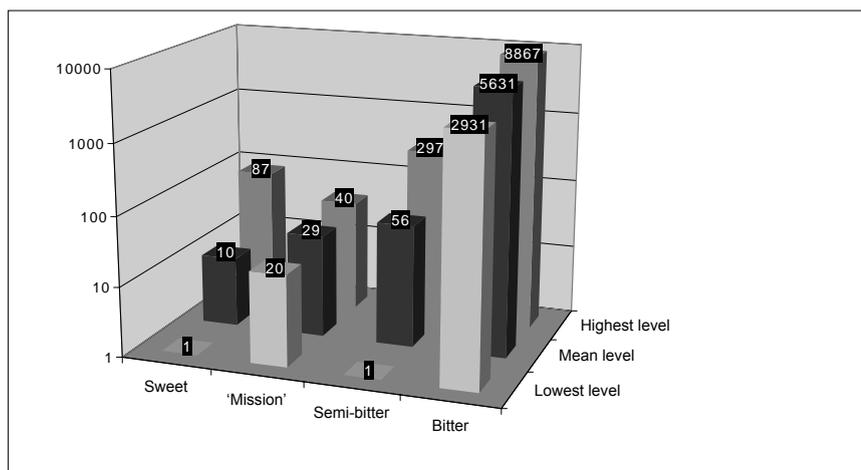


Fig. 1. Lowest, mean and highest amygdalin content (mg/100 g) in seeds of 26 sweet, 37 semi-bitter, 'Mission' and 14 bitter kernelled almonds. Lower limit of sweet and semi-bitter = 0, but for purposes of logarithmic scale the value 1 was used.

3. MS-Electronic nose and GC-MS analysis

Figure 2 shows the results of the MS-electronic nose analysis. The scores plot of the PCA model built to compare 50 almond genotypes and the female parent, 'Mission' shows a distinct separation of bitter kernels from semi-bitter and sweet kernels. 'Mission' grouped separately but close to semi-bitter.

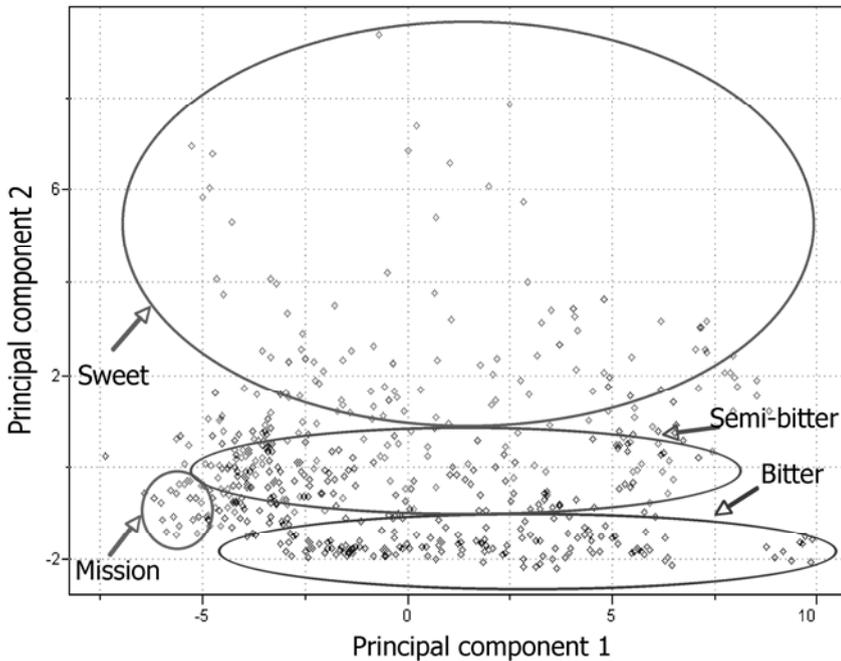


Fig. 2. Score plot of the first two principal components showing separation of sweet, semi-bitter, and bitter flavour groups in almond based on MS e_nose data.

The important fragment ions comprising the principal components with high loadings values in the almond kernels are 28 and 32 in factor one, and a combination of 44, 45, 27, and 32 for factor two. The data produced from the MS-electronic nose showed the abundance of fragment ion 44 was more than three times and two times greater in the sweet kernels compared with bitter and semi-bitter kernels, respectively. Fragment ion 45 was 2.5-times more abundant in sweet compared with bitter kernels. The GC-MS analysis provided verification of the data collected from the MS-electronic nose, as well as establishing the identity of the ions present. Extracted ion chromatograms (EIC) were used to identify the compounds responsible for ions 44 and 45.

From the EIC the major volatile component of sweet kernels was 2,3-butanediol at retention times of 7.08 min and 7.38 min (Fig. 3). The mass spectrum produced exact matches against authentic 2,3-butanediol and library spectrum (results not shown). The two retention times correspond to the racemic mixture of (D,L) 2,3-butanediol and the meso- form of 2,3-butanediol, respectively. The peak identifications were confirmed by analysing the authentic mixture of racemic/meso 2,3-butanediol on a chiral separating column. The ratio of racemic to meso-2,3-butanediol in the sweet sample was 3.72:1. This compound was detected in smaller quantities in the semi-bitter and bitter EIC at these retention times.

Major volatile components of semi-bitter and bitter almond kernels were determined to be benzaldehyde and benzyl alcohol. The mass spectra produced exact matches against authentic benzaldehyde and benzyl alcohol and library spectrum (results not shown). Benzaldehyde was present in significantly higher levels in bitter kernels than in semi-bitter and sweet kernels ($P < 0.001$; Table 1). Very low levels of benzaldehyde were present in sweet kernels. It was also found that benzyl alcohol was present in both bitter and semi-bitter kernels and at low levels in sweet kernels (Table 1). The fragment ions 27 and 28 from the MS-electronic nose could be due to hydrogen cyanide (HCN), benzaldehyde and/or benzyl alcohol as they are known fragment ions produced during mass spectrometry of these compounds.

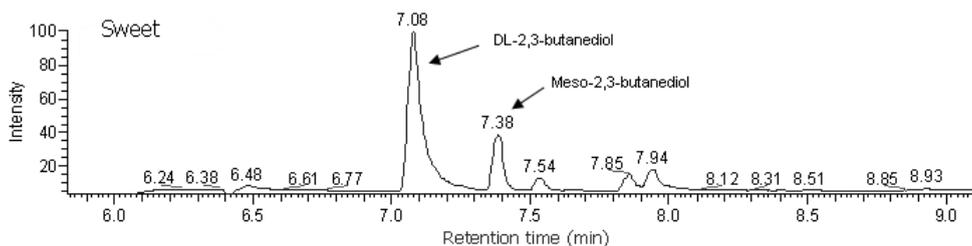


Fig. 3. GC-MS extracted ion chromatogram of mass 45 for sweet-kernelled almond.

Table 1. Volatile compounds of sweet, semi-bitter and bitter kernels of almond and their concentration in SPME/GC-MS

Flavour	(DL)-2,3-butanediol ($\mu\text{g g}^{-1}$) RT 7.08 min	Meso-2,3-butanediol ($\mu\text{g g}^{-1}$) RT 7.38 min	Benzaldehyde ($\mu\text{g g}^{-1}$) RT 7.02 min	Benzyl alcohol ($\mu\text{g g}^{-1}$) RT 10.31 min
Sweet	258.73	38.89	0.17	2.47
Semi-bitter	65.53	9.18	40.57	343.72
Bitter	11.61	0.88	37372.70	3381.81

RT = retention time.

As expected and confirmed by analysis, benzaldehyde (a product of amygdalin catabolism) was the major compound found in semi-bitter and bitter kernels, but the discovery of benzyl alcohol was unexpected and has not been reported previously. The formation of benzyl alcohol may be derived from benzaldehyde, via a reversible enzymatic reaction (Boatright *et al.*, 2004; Dudareva *et al.*, 2004). This may account for the low level of benzyl alcohol in sweet kernels, as the benzaldehyde content was also low. Benzyl alcohol could also be part of a separate pathway, which nonetheless may be important in almond flavour and aroma, as benzyl alcohol does impart an aromatic odor similar to benzaldehyde (Merck Co. Inc., 2001). Fragment ions 44 and 45 showed a greater abundance in sweet than semi-bitter or bitter kernels. These are known fragment ions of 2,3-butanediol. The presence of 2,3-butanediol in almond kernels has not been previously reported. This compound is also found in foods such as cheeses (Guillén *et al.*, 2004), honey (Soria *et al.*, 2005), and wine (Herold *et al.*, 1995) and its presence in sweet kernels may contribute to the characteristic taste often found in these kernels.

IV – Conclusions

The ability to separate almond flavours into sweet, semi-bitter, and bitter by chemical analysis, contributes to characterising the components of kernel flavour. Evidently amygdalin is not the sole determinant of kernel flavour. It would be interesting to analyse the taste threshold of 2,3-

butanediol using a taste panel. It potentially may be a factor in the sweetness of sweet cultivars. Using MS-electronic nose analysis we have confirmed the presence of benzyl alcohol and we have identified a second component (2,3-butanediol). The results validate this non-targeted characterization of metabolites to identify new chemicals linked to flavour phenotypes in almond kernels.

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