PICFPA	SAAPPA / SASPA / SAT	DFTS	Winetech
Canning Fruit Producers' Assoc. Submit to: Wiehahn Victor Tel: +27 (0)21 872 1501 inmaak@mweb.co.za	Fruitgro Science Submit to: Louise Liebenberg Tel: +27 (0)21 882 8470/1 louise@fruitgro.co.za	Dried Fruit Technical Services Submit to: Dappie Smit Tel: +27 (0)21 870 2900 dappies@dtd.co.za	Submit to: Jan Booysen Tel: +27 (0)21 807 3324 booysenj@winetech.co.za

			x

Indicate (X) client(s) to whom this final report is submitted.
Replace any of these with other relevant clients if required.

FINAL REPORT
FOR 2012

PROGRAMME & PROJECT LEADER INFORMATION

	Programme leader	Project leaders
Title, initials, surname	Dr. Hélène Nieuwoudt	Dr. Hélène Nieuwoudt ; prof Maret du Toit
Present position	Researcher	Researcher; Associate professor
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PROJECT INFORMATION

Project number	IWBT-B 08/10
Project title	Establishment of the metabolic profiles of lactic acid bacteria
Project Keywords	Malolactic fermentation, lactic acid bacteria, chemical analyses, wine aroma, sensory, consumer

Industry programme	**CFPA**	
	Deciduous	
	DFTS	
	Winetech	Microbiology committee
	Other	

Fruit kind(s)	Wine
Start date (dd/mm/yyyy)	01/01/2008
End date (dd/mm/yyyy)	31/12/2011

(Note: adjust footer – insert the project number no, researcher and research institution)

FINAL REPORT

(Completion of points 1-5 is compulsory)

1. Executive summary

Give an executive summary of the *total* project in no more than 250 words

This project investigated the contribution of industrial lactic acid bacteria (LAB) starter cultures to wine chemical composition, aroma and taste. The metabolic profiles associated with a selection of lactic acid bacteria (LAB) frequently used in the SA wine industry were established in experimentally produced Shiraz, Pinotage and Chardonnay wines.

An analytical platform consisting of methods that specifically include the detection and quantification of MLF-related volatile and non-volatile chemical compounds was established for this evaluation. The different LAB cultures showed significant strain-specific variations in citric acid degradation and lactic acid formation. Concentrations of compounds resulting from citric acid metabolism, diacetyl, acetic acid, acetoin, and ethyl lactate, were also affected, and depended on the bacterial strain used. Bacterial metabolism resulted in increased concentrations of higher alcohols, fatty acids, and total esters, with a larger increase in ethyl esters than in acetate esters. Formation of ethyl butyrate, ethyl propionate, ethyl 2-methylbutryate, and ethyl isovalerate was related to specific bacterial strains used, indicating possible differences in esterase activity.

Sensory evaluation of the wines by a trained panel showed that the chemical changes impacted the sensory profiles of the wines, particularly the fruitiness, vegetative and buttery attributes. Consumer preference tests showed that the changes imparted in sensory properties by the LAB bacteria, could also influence consumer liking. Selection of LAB starter cultures therefore has the potential to be used to influence the style of wine produced.

One PhD student were trained and one MSc is currently being trained chemical, sensory and consumer skills. The PhD student was employed by industry (Distell) as a sensory technologist.

2. Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

Malolactic fermentation (MLF) is essential for the aging of red wines and some white wines. During this process an important reaction is the conversion of malic acid to lactic acid, catalysed by lactic acid bacteria (LAB). This usually results in the acid stabilisation of wine, but the impact of the deacidification on sensory properties such as the flavour and texture of wine has been well-studied (Liu, 2000; Matthews *et al*., 2004). Several reports in the literature also suggest a contribution of LAB to wine aroma composition, however information on the latter aspect is fragmented and studies are mostly limited to a few selected chemical compounds such as diacetyl and dicarbonyl compounds (Bartowsky *et al*., 2004). The nature and concentrations of aroma compounds formed as a result of LAB metabolism remain largely unknown.

LAB have diverse genetic make-up and possess enzymes that can potentially covert pre-cursor compounds into aroma compounds including volatile phenols, esters, higher alcohols and acetic acid (Liu, 2002; Matthews *et al*., 2004). It is anticipated that the metabolism of LAB (and hence possible contribution) of LAB to wine aroma will be influenced by several factors including grape cultivar, concentrations of flavour precursors in grape juice, fermentation process technologies, maturation strategies and wine style. Although numerous studies have focused on the formation of grape- and yeast derived volatiles and the factors influencing their formation, the factors influencing LAB derived aroma compounds have not been studied in-depth. Based on the important impact that MLF has on the sensory

properties of wine, it is of high importance to investigate the contribution of industrial LAB starter cultures on the volatile composition of wine.

This project has two aims, namely the development of a portfolio of analytical methods suitable for the identification and quantification of mostly, but not exclusively, aroma compounds in wine and to apply these methods to evaluate the contribution of selected LAB starter cultures to wine aroma and flavour. The analytical method development will have a particular focus on rapid high-throughput analytical strategies suitable for detailed metabolic profiling. Experimental wines will be produced using various combinations of yeast and LAB starter cultures under different winemaking conditions. The optimised analytical methods will then be used to do wide-scale chemical analyses of the wines. Wines will also be subjected to sensory evaluation. Statistical correlations will be sought between chemical and sensory data with the ultimate aim to evaluate the contribution of MLF to wine aroma and flavour. The information gained will be used to formulate a better understanding of the integrated yeast/LAB contribution to the establishment of wine aroma.

3. Workplan (materials & methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

Project 1a: Optimisation of methods for sample preparation and development of analytical methods for detection and quantification of aroma compounds

Milestone 1: Evaluation of solid phase extraction (SPE) procedures for sample cleanup and analyte concentration as well as evaluation of headspace sampling using selected fibres for solid phase micro extraction (SPME) and GC-MS analysis

Task 1: Test and optimise experimental parameters for sample preparation for GC-MS including optimal extraction time and temperature and desorption time and temperature for various fibres. Fibres evaluated will include PDMS/CAR; PDMS/DVB and PDMS/DVB/CAR.

Task 2: Optimise conditions for GC analysis and separation of analytes through variation of GC operating conditions including flow rate of carrier gas, splitflow and temperature conditions in the GC oven.

Task 3: Apply the developed analytical strategies to different wine styles and redevelop suboptimal techniques so that a portfolio of strategies are available for extended metabolic profiling of aroma compounds relevant to this study.

Project 1b: Establishment of high-throughput analytical methods for aroma compounds

Milestone 1: Introduction of fast GC, optimisation of sample preparation and method development for analysis of aroma compounds

Task 1: Re-development of our existing GC-FID liquid-liquid extraction method that analyses 27 volatile compounds to increase the speed of analysis. High pressure narrow-bore column technology in combination with method translation (www.agilent.com) will be used. This will theoretically, at least double the sample throughput for this method.

Task 2: Apply the developed analytical strategies to different wine styles and redevelop suboptimal techniques. Establish a portfolio of optimised fast GC methods.

Project 1c: Application of high-throughput analytical methods for non-volatile compounds

Milestone 1: Optimisation of capillary electrophoresis (CE) for analysis of amino acids and organic acids

Task 1: Full implementation of CE for analysis of amino acids and organic acids with the aim of establishing high-sample throughput and high speed of analysis.

Task 2: Application of the developed methods to different wine styles and redevelopment of suboptimal techniques.

Project 2a: Metabolic profiling of experimental wines produced with different LAB starter cultures under standardised winemaking conditions

Milestone 1: Establishment of comprehensive metabolic profiles associated with the LAB starter cultures by analysing small-scale experimental wines produced under standardised winemaking conditions

Task 1: Small-scale wine fermentations with selected commercial LAB starter cultures. Both red (Cabernet Sauvignon, Pinotage, Shiraz) and white wines (Chardonnay) will be made according to standard winemaking practices. Different inoculation strategies of co-inoculation and consecutive inoculation will be used.

Task 2: Investigation into the variability associated the aroma profile of LAB bacteria used in the winemaking and the exploration of possible correlations between LAB starter culture and aroma profile.

Task 3: Metabolic profiling of fermentations through application of the analytical methods developed for aroma and non-volatile compounds. Chemical data will be captured in an electronic database and subjected to intensive statistical analysis, using both univariate techniques (ANOVA, distribution profiles) as well as multivariate data analysis techniques including principal component analysis (PCA) and partial least squares regression discriminant analysis (PLSD).

Task 4: Wines will be analysed using a trained sensory panel and data will be analysed with multivariate data techniques to investigate possible correlations between sensory and chemical data. The effect of MLF selection on consumer liking will also be evaluated. Based on the outcomes of these results, LAB starter cultures showing interesting metabolic profiles will be used to evaluate the influence of winemaking practices on the aroma profile of LAB cultures as set out under project 2b.

Project 2b: Evaluation of the influence of winemaking practices on the production of aroma compounds by selected yeast/LAB combinations

Milestone 1: Evaluation of the main effects on the expression of the aroma profile of LAB bacteria using an experimental design approach

Task 1: Production of small-scale experimental wines to evaluate the important physical and chemical parameters that have the most prominent impact on the metabolic profile of selected yeast/LAB combinations. For this project an experimental design will be used with the aim to develop a model system suitable for evaluation of main effects (referring in this context to winemaking conditions) by using the minimum number of necessary chemical

analyses. The inoculation strategy that allows for "optimal" expression of the aroma profile of the LAB cultures (as established under Project 2a) i.e. co-inoculation or consecutive inoculation will be used in this project. Wines will be evaluated by sensory analysis.

Milestone 2: Evaluation of the relative contribution of LAB to the aroma profiles of specific wine styles and investigation of possible correlations between LAB and aroma profiles of these wines

Task 1: Chemical and sensory data that have been generated will be subjected to detailed statistical analysis in order to investigate possible correlations between LAB aroma profile and the sensory properties of wine.

Milestone 3: Evaluation of the influence of South African malolactic bacteria on wine aroma, using strains from the LAB culture collection of IWBT

Task 1: Monitoring the progress MLF through the development of multivariate statistical process control charts based on FTNIR and FTMIR spectra
Task 2: Assessing the impact of the strains on the volatile and non-volatile aroma compounds
Task 3: Correlating the aroma compound production to sensorial descriptors

4. Results and discussion

State results obtained and list any benefits to the industry. Include a short discussion if applicable to your results. This final discussion must cover ALL accumulated results from the start of the project, but please limit it to *essential* information.

Project and Results	Achievements and industry benefits
Optimisation of methods for sample preparation and development of analytical methods for detection and quantification of aroma compounds (Project 1a, Milestone 1)	Achievements: Sample cleanup and concentration methods were developed. GC-FID (39 compounds) and GC-MS (48 compounds) methods were optimised. Methods were used to do chemical profiling of experimental Shiraz, Pinotage and Chardonnay wines. **Industry benefits:** Analytical platform using modern state of-the-art technologies (sample preparation, detection and quantification) for aroma compounds in wine was established. These technologies, and those in the following sections, were also transferred to industry through application of the methods in routine analysis of (i) experimental samples in other concurrently running Winetech funded projects of IWBT-DVO, (ii) ARC Infruitec projects and, (iii) wines of private producing cellars.

Establishment of high-throughput analytical methods for aroma compounds (Project 1b, Milestone 1)	Achievements: Fast GC method established and validated: 39 compounds. Run time for chromatographic separation reduced from 45 to 15 minutes. Industry benefits: Increased sample throughput method developed for aroma compounds in wine: three-fold reduction in analysis time achieved.
Establishment of high-throughput analytical methods for non-volatile compounds (Project 1c, Milestone 1)	Achievements: CE method for 7 organic acids developed by dr A. Buica (DVO, US). Not part of project IWBT-B 08-10. Industry benefits: Increased sample throughput method developed for profiling of organic acids in wine.
Metabolic profiling of experimental wines produced with different LAB starter cultures under standardised winemaking conditions (Project 2a, Milestone 1) **Evaluation of the influence of winemaking practices on the production of aroma compounds by selected yeast/LAB combinations (Project 2b, Milestone 1, 2,)**	Achievements: Chemical profiles of Shiraz ad Pinotage wines elaborated with *S. cerevisiae* WE372 and *O. oeni* cultures Enoferm alpha, Lalvin VP41, Viniflora oenos and Viniflora CH16, were established, using sequential fermentation strategies. Chardonnay was produced with *S. cerevisiae* NT202 and *O. oeni and L. plantarum* LAB, using co-inoculation and sequential fermentation strategies. Industry benefits: New insights into the chemical profiles associated with LAB cultures frequently used in the SA wine industry was generated in red and white wine cultivars. Analytical platform can be used to screen newly developed starter cultures. The technologies are used in related IWBT-DVO research projects.
Evaluation of the contribution of South African malolactic bacteria to the sensory profile and consumer preference of Pinotage and Shiraz culture collection of IWBT (Project 2b, Milestone 3) Comment 24 April 2012: Consumer preference analysis was not planned in the original proposal, but added later as the project evolved.	Achievements: Differences in buttery and fruity sensory attributes were found and were more pronounced in Shiraz than Pinotage. Consumer preferences showed LAB starter culture could have an effect on liking of red wine. Industry benefits: Sensory panel for MLF was established, that can be contracted to do testing in MLF related projects from industry. Consumer preference mapping techniques (statistical tools) were established and the technology transferred to industry through training of post graduate students.

	PanelCheck and Compusense software packages required in sensory analysis were mastered. These skills were transferred to industry through training of the post graduate students. Multi-skilled students (chemical, sensory and consumer) have been trained. The PhD graduate is already employed as sensory analyst by industry.
Development of 2D-GC method for comprehensive profiling of MLF-related aroma compounds in Pinotage wine. Comment 24 April 2012: This extension, not funded by Winetech, was not included in the original proposal, but formulated based on the achievements. Industry benefits are listed.	Achievements: Comprehensive 2D-GC gas chromatography of Pinotage wines produced in project IWBT-B 08-10 was done. The data handling protocols were optimised. Benefits to industry: Improved understanding of the chemical changes imparted by LAB bacteria in Pinotage was achieved.

5. Accumulated outputs

List ALL the outputs from the start of the project.
The year of each output must also be indicated.

Research outputs

- Elaboration of Shiraz and Pinotage wines with four different *O. oeni* LAB cultures: 2008 and repeated in 2009
- SPE and SPME sample preparation methods developed: 2008
- GC-FID and GC-MS methods for wine aroma and MLF compounds developed: 2008
- Analysis of Shiraz and Pinotage wines for aroma compounds and organic acids: 2008 and 2009
- Sensory tests: training of panel for quantitative descriptive analysis 2009
- Consumer preference tests 2009
- 2D GC analysis and establishment of data handling protocol: 2010 and 2011
- Elaboration of Shiraz wines with five different *O. oeni*, L. p*lantarum* LAB cultures, and using co-inoculation and sequential fermentation strategies: 2010
- GC-FID analysis of fermentation compounds of 2010 Shiraz wines: 2011
- Sensory analysis of 2010 Shiraz wines: 2011
- Elaboration of Chardonnay wines with four different *O. oeni* LAB cultures: 2010
- GC-MS of Chardonnay wines: 2010

Presentations/Conference proceedings: 16

- National: 8
- International: 6
- Workshops: 2

Publications

Scientific publications (peer reviewed): 6

- Published (2011 and 2012): 2
- Submitted (2012): 1

- Under preparation for submission in 2012/2013: 3

Technology development, products and patents

Indicate the commercial potential of this project (intellectual property rights or a commercial product(s)).

Technology development

- SPE and SPME sample preparation methods optimised for wine aroma and MLF compounds
- GC-FID and GC-MS methods for determination of for wine aroma and MLF compounds optimised
- Fast GC-FID method for high sample throughput for wine aroma profiling developed
- CE method for high-throughput screening of organic acids established
- Trained sensory panel for MLF established
- Competences in sensory quantitative descriptive profiling tests established
- Competences in consumer preference profiling methods established f

Human resources development/training

Indicate the number and level (e.g. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

Student level (BSc, MSc, PhD, Post doc)	Cost to project (R)
Sulette Malherbe – PhD graduated March 2011	1,500
Jessica Garlick – MSc. Second year MSc; due for graduation December 2012.	20,000

Publications (popular, press releases, semi-scientific, scientific)

Scientific

(1) Malherbe, S., A.G.J. Tredoux, H.H. Nieuwoudt & M. du Toit. **2012**. Comparative metabolic profiling to investigate the contribution of *O. oeni* MLF starter cultures to red wine composition. *J. Ind. Microbiol. Biotechnol.* 39: 477-494.

(2) Vestner, J., S. Malherbe, M. du Toit, H.H. Nieuwoudt, A. Mostafa, T. Górecki, A.G.J. Tredoux & A.J. de Villiers. **2011**. Investigation of the volatile composition of Pinotage wines fermented with different malolactic starter cultures using comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF-MS). *J. Agric. Food Chem.* 59: 12732-12744. (not funded by Winetech)

(3) Malherbe, S., E. Menichelli, M. du Toit, A. Tredoux, N. Muller, T. Næs & H. Nieuwoudt. **2012**. The effect of malolactic fermentation using four *Oenococcus oeni* as starter cultures on the chemical composition, sensory properties and consumer liking of Pinotage wines. *Food Res. Int.* (Submitted).

(4) Malherbe, S., H.H. Nieuwoudt, M. du Toit & A.G.J. Tredoux. **2012**. High throughput quantification of major volatile compounds in wine: fast GC method development, validation and application. *Food Chem.* (In preparation).

(5) Nieuwoudt *et al*., Monitoring of evolution of aroma compounds in Chardonnay wines elaborated with different lactic acid bacteria and inoculation strategies, using FT-IR spectroscopy and GC-MS (preliminary title, manuscript under preparation. This output is also listed in Winetech report IWBT-B 08-11: Bioprocess monitoring FINAL-REPORT 2012).

(6) Garlick *et al*., Comparative profiling of the effects of *O. oeni* and *L. plantarum* starter cultures and inoculation strategies on the chemical and sensory profiles of Shiraz wine (preliminary title, manuscript to be prepared end 2012/start 2013).

Presentations/papers delivered

1. Malherbe, S., Roux, K., Nieuwoudt, H.H., Du Toit, M. and Tredoux, A.G.J., **2008.** Increasing sample throughput by analysing major volatile compounds in wine with fast GC-FID. 31st SASEV Conference, 11-14 Nov 2008, Somerset West, South Africa.
2. Malherbe, S., Tredoux, A.G.J., Du Toit, M. and Nieuwoudt, H.H., **2008**. Evaluating the impact of different MLF starter cultures on wine aroma and flavour: a holistic/multivariate approach. 31st SASEV Conference, 11-14 Nov 2008, Somerset West, South Africa.

3. Malherbe, S., Nieuwoudt, H.H., Du Toit, M. and Tredoux, A.G.J., **2008**. Determination of MLF related carbonyl compounds with the use of headspace SPME GC-MS. 31st SASEV Conference, 11-14 Nov 2008, Somerset West, South Africa.
4. Malherbe, S., Tredoux, A.G.J., Du Toit, M. and Nieuwoudt, H.H., **2008**. Evaluating the impact of different MLF starter cultures on wine aroma and flavour: a holistic/multivariate approach. South African Chemometric Society Seminar, 1-5 Dec 2008, Stellenbosch, South Africa.
5. Malherbe, S., Nieuwoudt, H.H., Du Toit, M. and Tredoux, A.G.J., **2009**. Analysis of carbonyl compounds during MLF in red wine: application of SPME GC-MS. In Vino Analytica Scientia, 2-4 July 2009, Angers, France.
6. Malherbe, S., Tredoux, A.G.J., Du Toit, M. and Nieuwoudt, H.H., **200**9. MLF definitely makes a difference: the aroma facts. 4th International SASEV Conference, 28-30 July 2009, Cape Town, South Africa.
7. Malherbe, S., Nieuwoudt, H.H., Du Toit, M. and Tredoux, A.G.J., **2009**. Making it faster: evaluation of a fast GC-FID method for wine volatile analysis. 4th International SASEV Conference, 28-30 July 2009, Cape Town, South Africa.
8. Malherbe, S., Nieuwoudt, H.H., Du Toit, M. and Tredoux, A.G.J., **2009**. Analysis of carbonyl compounds during MLF in red wine: application of SPME GC-MS. 4th International SASEV Conference, 28-30 July 2009, Cape Town, South Africa.
9. Malherbe, S., Nieuwoudt, H.H., Du Toit, M. and Tredoux, A.G.J., **2009**. A simple method for high throughput analysis of volatiles in wine. In Vino Analytica Scientia, 2-4 July 2009, Angers, France.
10. Malherbe, S., Tredoux, A.G.J., Du Toit, M. and Nieuwoudt, H.H., **2009**. Evaluation of the potential contribution of lactic acid bacteria to wine aroma and flavour. 4th International SASEV Conference, MLF Workshop: Beyond Deacidification – Aroma possibilities and variations, 28-30 July 2009, Cape Town, South Africa.
11. Du Toit, M., C. Knoll, S. Malherbe, E. Lerm, L. Engelbrecht, J. Carstens, S. Mtshali & D. Rauhut. **2010**. Impact of lactic acid bacteria on wine aroma. International *Intervitis Interfructa* Congress [60th German Grape and Wine Congress], New Stuttgart Trade Fair Centre, Germany.
12. Malherbe, S., H.H. Nieuwoudt, M. du Toit & A.G.J. Tredoux. **2010**. Metabolic profiling of volatiles produced during MLF fermentation: application of SPME GC-MS. Cape Biotechnology Forum 2010, Lord Charles Hotel, Somerset West.
13. Malherbe, S., H.H. Nieuwoudt, M. du Toit & A.G.J. Tredoux. **2010**. A simple method for high throughput analysis of volatiles in wine. Cape Biotechnology Forum 2010, Lord Charles Hotel, Somerset West.
14. Malherbe, S., T. Naes, A.G.J. Tredoux, M. du Toit & H.H. Nieuwoudt. **2010**. Impact of malolactic fermentation starter cultures on red wine aroma compounds, sensory properties and consumer preference. Thirty Second Conference of the South African Society for Enology and Viticulture, Lord Charles Hotel, Somerset West.
15. Du Toit, M. **2011**. How does MLF impact on wine aroma? Lallemand-IWBT MLF workshop, Stellenbosch University, Stellenbosch.
16. Garlick, J., M. du Toit & H. Nieuwoudt. **2012**. Chemical and sensory attributes of Shiraz wines produced by different lactic acid bacteria and using different inoculation strategies for malolactic fermentation. In preparation for 34th SASEV Conference, Stellenbosch, 14-16 November 2012.

4. Total cost summary of project

	Year	CFPA	Deciduous	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2008				R 100,000	R 47,500		**R 147,500**
Total cost in real terms for year 2	2009				R 110,000	R 55,000		**R 165,000**
Total cost in real terms for year 3	2010				R 100,000	R 50,000		**R 150,000**
Total cost in real terms for year 4	2011				R 100,000	R 50,000		**R 150,000**
Total cost in real terms for year 5	2012				R 0	R 0		**R 0**
TOTAL					**R 410,000**	**R 202,500**		**R 612,500**