

Grape and Wine
Research and
Development Corporation

Development of biosensor for the detection of 2,4,6-trichloroanisole, a major contaminant of corks and wines

FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CST 1GW**

Principal Investigator: **Dr D Barnett**

Research Organisation: **CSIRO**

Date: **1992**

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RURAL INDUSTRY RESEARCH FUNDS
CONTINUING* PROJECT APPLICATION
FINANCIAL YEAR 1992/93

Authorised Body:	GRAPE AND WINE RESEARCH COUNCIL	
Project Number:	CST1V	
Project Title:	Development of a biosensor for the detection of 2,4,6-trichloroanisole a major contaminant of corks and wines	
Field of Research:	Wine	**Field Code:** 7
Organisation: **Address:**	CSIRO PO Box 225 Dickson ACT	**Postcode:** 2602
Project Supervisor:	Professor D.G. Laing	
Division	University of Western Sydney	**Telephone:** (045) 701 333
Branch:	Faculty of Science & Technology	**Facsimile:** (045) 701 383
Address:	Bourke Street Richmond NSW	**Postcode:** 2753
Research Staff:	See Annexure	
Administrative Staff:	Mr G.W. Goodfellow CSIRO Division of Food Processing	**Telephone:** (02) 887 8333 **Facsimile:** (02) 887 3107
Anticipated Commencement Date:	1.7.89	**Anticipated Completion Date:** 31.12.92
Funds Provided/Sought From Other Sources:	The CSIRO contribution (salary, operating and general overheads) to the project is estimated to be $166,000 in 1991/92. The ARC have provided $12000 (1989-90) for Stage 1 of this project. University of Western Sydney contribution (in kind), Professor Laing's salary is $15000.	
Aims:	To develop a device that will detect and measure 2,4,6-trichloroanisole, a major odorous contaminant of corks and wines	
Industry Significance:	The wine industry is threatened with losses of millions of dollars as a consequence of the contamination of wines by corks containing 2,4,6-trichloroanisole. Since no simple objective method exists for detecting this substance, this project aims to develop a highly specific and sensitive sensor that can be used at the vineyard and which will provide a cheap, simple and rapid analysis of this substance, thereby significantly reducing the chance of producing contaminated wines and loss of revenue.	

BUDGET:	Salary $	Travel $	Operating $	Capital $	Total $
Approved 1990/91	10000		5000		15000
Jul-Dec	12500		2500		15000
Jan-Jun	12500		2500		15000
1991/92	25000		5000		30000
1992/93	10000		2200		12200

Estimated Project Income: Royalties from manufacture of device

Research Proposal Summary:

2,4,6-Trichloroanisole in corks is a major contaminant of wines and a source of lost revenue to producers. Since there is no immediate solution to overcoming this problem and since it is an irregular occurrence, there is a need for a sensitive but simple device that can be used by wine producers at the vineyard to determine the quality of corks and levels of the substance in wines.

Recently this Division initiated research aimed at producing sensors for detecting and measuring levels of desirable and undesirable components in foods and beverages during processing. Since 2,4,6-trichloroanisole is a common contaminant of foods, a project was initiated to develop a sensor to detect and measure low levels of this substance. The type of sensor being developed will be suitable for detecting trichloroanisole in corks and wines as indicated in the original grant. The present grant request is for funds for Stage 3 of this three stage project. Stage 1 was funded by an ARC grant with the University of Sydney. Stage 2, the topic of grants from the Grape & Wine Council has been in progress for 18 months. Stage 3, the integration of the biological and electronic components of the sensor is scheduled to commence in July 1991.

Animal Experimentation

I hereby certify that the project in this application has been examined by the animal experimentation ethics committee of the North Ryde site.

I agree to observe all requirements set out in the national "Code of Practice for the Care and Use of Animals for Experimental Purposes".

Signature of Project Supervisor: [signature] Date: 17/2/92

ANNEXURE

Staff

Principal Investigators

1. Professor D.G. Laing, Professor of Food Technology (5%)
2. Dr D. Barnett, Principal Research Scientist (CSIRO Food Processing) (20%)
3. Dr J. MacFarlane, Principal Research Scientist (CSIRO Applied Physics) (20%)
4. Mr C. Clark, Experimental Scientist (CSIRO Food Processing) (30%)
5. Mr P. Dencher, Technical Officer (CSIRO Applied Physics) (20%)
6. Dr M. Lake, Research Fellow (CSIRO Applied Physics) (20%)

ATTACHMENT A

A. SALARIES

1. Staff costs	1992/93 $
Experimental Officer (Biochemist) (part-time)	5000
Technical Officer (Electronics) (part-time)	5000
Total Salary	10000

B. OPERATING COSTS

Chemical, animals, animal food, maintenance, labware	2200
Total Requested	12200

BUDGET JUSTIFICATION

Staff:

Mr C. Clark, a part-time employee of CSIRO with experience in the synthesis and purification of odour sensing antibodies, will spend 30% of his working time on this project. His appointment is necessary for the continuing preparation, purification and characterisation of the specificity of antibodies for sensing 2,4,6-trichloroanisole.

Mr Peter Dencher, an electronics engineer from the Sensor Technology Group of the CSIRO Division of Applied Physics is working on the transduction and measurement of electrical changes in piezoelectric wafers e.g. capacitance, resistance, resulting from the interaction of 2,4,6-trichloroanisole with appropriate antibodies on the surface of the wafer. He is working closely with the staff of the Food Research Laboratory to develop an antibody based electronic sensor. The funds requested for this position are to cover, in part, the time he will specifically work on the 2,4,6-trichloroanisole sensor. The project will benefit, however, from the information obtained in developing sensors with other antibodies he is working on.

The chemicals and laboratory ware are required for the preparation and characterisation of odour-protein conjugates, and antibodies. Also essential are animals, animal food and animal housing.

The additional funds for an extension of the project are sought to provide what appears now to be the time required to fully test the antibodies bound to the piezoelectric wafers. The difficulties we have encountered in characterising the antibodies have cost us time and the full potential of the work should be the realised with the extension of 6 months.

1991/92 ESTIMATE

ITEM	PREVIOUS $	CURRENT $	1991/92 $	1992/93 $
A. SALARIES				
Staff Costs				
Mr C. Clark Experimental Scientist	9000	9000	8000	4500
Mr P. Dencher Technical Officer			12000	4500
Other Salary Costs				
Payroll Tax	1000	1000	5000	1000
Worker's Insurance				
Superannuation				
Other (specify)				
TOTAL SALARIES	10000	10000	25000	10000
B. TRAVEL (Itemise Fares, Personal Allowances)				
TOTAL TRAVEL				
C. OPERATING				
Chemicals, labware	2500	3000		1400
Animals, animal feed and housing	500	2000		800
TOTAL OPERATING	0	5000		2200
D. **CAPITAL**				
TOTAL CAPITAL				
TOTAL REQUESTED	13000	15000	30000	12200

ATTACHMENT B

RESEARCH PROPOSAL

Objective

Contamination of wines by corks containing the musty odorant 2,4,6-trichloroanisole is a major cause of lost revenue to wine producers. Although the underlying cause of contaminated corks is understood, there is no obvious and immediate solution to the problem. Identifying and avoiding use of contaminated corks, however, will reduce the loss to producers. The objective of this project is to construct a cheap and sensitive device for detecting and measuring the quantity of 2,4,6-trichloroanisole in corks and thereby minimize use of contaminated corks. The device will also measure the contaminant in wines and provide a further check of wine quality before purchase by the consumer.

Staff

1. Professor D.G. Laing, Professor of Food Technology	(5%)
2. Dr D. Barnett, Principal Research Scientist	(20%)
3. Dr J. MacFarlane, Principal Research Scientist	(20%)
4. Mr C. Clarke, Experimental Scientist	(30%)
5. Mr P. Dencher, Technical Officer	(20%)
6. Dr M. Lake, Research Fellow	(20%)

Facilities

This Division possesses a wide range of chemical and biochemical facilities that are required for the project. Animals for this project are currently housed at the adjacent Division of Animal Production. Electronics facilities for the design, construction and testing the electronic sensor are available at the CSIRO Division of Applied Physics and this Division.

Background

2,4,6-Trichloroanisole is a musty odorant produced by the metabolism of 2,4,6-trichlorophenol which is a product of microorganism attack on chlorinated substances used to sterilise corks.

Although a number of substitutes for corks are available none have all the desirable characteristics of cork.

Use of other sterilants has also been tried but none have proven totally satisfactory. Thus a solution remains to be found and it can be expected that 2,4,6-trichloroanisole will continue to be a problem for wine producers for the foreseeable future.

Of considerable value to producers would be a device which would measure the level of the contaminant in corks, and as a further precaution, also in wines. Action in-house

could then be taken either to avoid use of contaminated corks or delivery to consumers of tainted wines, thus reducing both revenue losses and litigation.

Accordingly, the ideal device for detecting contaminated corks would be capable of being sited in small or large vineyards, be cheap, portable, simple to use, and highly specific and sensitive to 2,4,6-trichloroanisole. Such a device could be operated by non-scientific staff and thereby avoid the need for corks or wine samples to be tested elsewhere at a standard laboratory. Current measurement techniques for trichloroanisole require the use of expensive gas chromatographs, and often mass spectrometers are needed for unequivocal identification. Although gas chromatographs are not uncommon in the laboratories of larger wine producers, mass spectrometers are rarely seen at vineyard laboratories.

The objective of this project is to construct such a device. The basic conceptual design of the device, commonly known as a biosensor (Van Brunt, 1987), is simple. A biological receptor such as an antibody (Bush & Rechnitz, 1987), enzyme (Frew & Hill, 1988) or protein, is layered onto the surface of an electronic transducer that may already be coated with a conducting membrane or film. Biosensors are already in use for measuring glucose in fermentation processes (Van Brunt) and a host of others are in different phases of construction varying from portable detectors for farmers to test the level of pesticides in their soil, to on-line measurement of the potassium content of blood during surgery (Laing, 1989). Biosensors display several unique features that make them attractive. They are small (pen-size), simple to use, and require no additional reagents as in chemical analyses. They are portable, inexpensive and perfect for remote or automated sensing and data processing. For example, they could be used for automated analyses of large numbers of cork or wine samples.

Industry Significance

Contaminated wine resulting from contact with corks containing 2,4,6-trichloroanisole is threatening to cost wine producers millions of dollars in lost revenue and reputation. Development of a device which will identify contaminated corks or wines, will significantly reduce the chance of producing contaminated wines and avoid the subsequent adverse publicity and litigation that can ensue. Furthermore, since the device will measure not just detect odours, an objective measurement of offensive odour levels can be recorded if necessary, and can be used to the producers advantage in litigation cases.

Methodology

The aim of this project is to construct a sensor for 2,4,6-trichloroanisole consisting of an antibody that specifically recognises this substance and is bound to the surface of an electronic transducer. The antibody will be inserted into a conducting film and the whole layered onto the surface of the electronic transducer.

The project, already underway consists of three stages. In Stage 1 which was funded by a CSIRO-University of Sydney Australian Collaborative Research grant and an Australian Research Council Grant, conjugates of 2,4,6-trichloroanisole and bovine serum albumin of different configurations, were synthesized. In Stage 2 (current), antibodies to these

antigens are being prepared, purified and characterised. Currently two antibodies that are sensitive to 2,4,6-trichloroanisole are being purified. An enzyme-linked immunoassay (ELISA) is under development to establish the specificity and sensitivity of both. It is possible that a simple version of this assay called a 'dipstick' may be useable as a rapid assaying technique for the contaminant during 1991.

In the third stage of the project, the subject of this grant request, funds are required for two purposes:

1. The development of methods for attaching the antibody for 2,4,6-trichloroanisole to a quartz (piezoelectric) wafer and for transducing and measuring the electrical changes produced when the odorant binds to the antibody.

2. The continued production and purification of the antibody to provide sufficient product for (1) to be achieved.

Associated Activity

The proposed sensor is one of several targeted for construction by the Chemical Sensors Group of the CSIRO Division of Food Processing, the Sensor Technology Group of the CSIRO Division of Applied Physics, and the Surface Chemistry Group of the CSIRO Division of Chemicals and Polymers. Research with antibodies is also being conducted by the Food Group in collaboration with several University groups on other sensors which will add to the chances of success of the present proposal. The Group has been successful in obtaining several collaborative grants and an active program is under way. In one of these a prototype sensor based on an antibody as the sensing molecule, has been developed for an artificial sweetener and others are being developed for a food toxin and environmental odours.

Publication and Dissemination

Dissemination of information through direct contact with industry, primary industry newsletters and industry related journals will be the major mode of communication with the industry. Sale of the sensor to the wine and food industry worldwide will be handled by an industry partner.

Other Comments

1. The Chemical Sensor Group of the Division of Food Processing consists of experts from biochemistry, chemistry, neurobiology and olfaction. It was established to channel the talents of scientists from different disciplines onto a specific target - chemical sensors. More recently this Group has combined with others in CSIRO with expertise in electronics and surface chemistry. The expertise gathered in this powerful group is probably unique in the world.

2. Development of a sensor for 2,4,6-trichloroanisole will help provide the technology necessary for the construction of other sensors for on-line monitoring of wine manufacture and quality control.

3. This document is STRICTLY CONFIDENTIAL.

References

Bush, D.L & Rechnitz, G.A. (1987). Monoclonal antibody biosensor for antigen monitoring. Anal.Lett., 20:1781.

Van Brunt, J. (1987). Biosensors for bioprocesses. Biotechnol., 5:437.

Frew, J.E. & Hill, H.A.O. (1988). Direct and indirect electron transfer between electrodes and redox proteins. Eur.J.Biochem., 172:261.

Kulp, T.J., Camins, I., Angel, S.M., Munkholm, C. & Walt, D.R. (1987). Anal Chem. 59:2849.

Laing, D.G. (1988). Biosensors and the food industry. Food Aust. 41:799-801.

ATTACHMENT C

PROGRESS REPORT 1991-92

Four modified 2,4,6-trichloroanisole molecules, designed by CSIRO, have been synthesized by collaborators at the Department of Organic Chemistry, University of Sydney. These molecules have 'spacer' groups attached to 2,4,6- trichloroanisole, the 'spacer' groups being used to bind the anisole to the protein, bovine serum albumin (BSA).

These 2,4,6-trichloroanisole-protein conjugates are needed because the odorant by itself is too small to induce an adequate antibody response in animals. Each molecule of BSA can have up to 30 molecules of odorant bound to it providing a significant number of sites for a specific antibody response to the 2,4,6- trichloroanisole portion of the conjugate.

During the first 12 months of the project, three 2,4,6-trichloroanisole-protein conjugates (A, B and C) were synthesized, while the fourth (D) was synthesized recently (see earlier Progress Reports). Sufficient quantities of these antigens have been synthesized to ensure an adequate quantity of material for antibody production and sensor development. In addition, the corresponding phenol precursors have been synthesized as these are also widely-found tainting substances. Antibodies for these substances will be sought at some later date not necessarily associated with the present grant.

Two of the trichloroanisole-BSA conjugates B and C (see earlier Report) were injected into rabbits to produce antibodies to the odorant. The injection programs which require many months are continuing and have produced antibodies to both conjugates that recognise 2,4,6-trichloroanisole. Both antibodies are currently being purified and their specificity and sensitivity characterised.

Production of antibodies to antigens A and D (see earlier Report) has not commenced due to the concentration of effort into purifying antibodies to B and C. The task of purifying the latter two antibodies has proven to be more difficult than anticipated because of the difficulty in preparing affinity gels to which are attached the odorant. These gels are commonly used to remove, through binding, the required antibody from rabbit serum. However, the succinimide ester of 2,4,6-trichloroanisole which is used to attach the odorant to the gel, is insoluble in aqueous media necessitating the use of other solvent systems many of which degrade the gel. Another approach used in an attempt to circumvent the above solubility problem involved the use of Protein A affinity gels. Although we have had some success with this method consistent results have not been obtained. Other methods of purification are continuing to be investigated. However, it is possible that direct attachment of the semi-purified antibody to a piezoelectric device (see below) may prove to be the more successful approach. This approach has been used by others to produce a biosensor.

Development of a reliable ELISA test (immunoassay) to evaluate the specificity and sensitivity of the TCA antibodies has been significantly retarded because of solubility problems with the antigens. In brief, although occasional satisfactory dose-response curves have been obtained, loss of TCA from solution through precipitation and adsorption onto container surfaces has made it difficult to satisfactorily test the characteristics of the antibodies.

Another more direct approach which we are now in the process of gearing up for, is as mentioned above, to bypass the development of the standard immunoassay test and test the specificity and sensitivity of the antibodies directly attached to piezoelectric wafers of our recently developed electronic transducers (see below). This approach was successfully used for the development of pesticide biosensors, e.g., parathion.

A major advance during the past year has been the successful development of two prototype piezoelectric based electronic transducers to which we aim to attach the above antibodies. One of the prototypes is a dual detector device, one detector compensating for humidity, the other measuring the quantity of target odorant. The stability and sensitivity of these electronic devices in different temperature and humidity conditions provides state of the art transduction processes for the proposed TCA biosensor. Considerable work has been in progress in determining the optimum conditions for attaching antibodies to the piezoelectric wafers in an effort to maximise the amount of antibody that will attach to the surface in an optimal orientation to sense the odorant.

In summary, two antibodies sensitive to TCA have been prepared and partly purified. Work is continuing on the development of methods to overcome solubility and adsorption problems for the development of a suitable assay to characterise the specificity and sensitivity of the antibodies.

Two prototype piezoelectric based transducers have been constructed and the development of methods for attaching antibodies to the piezoelectric wafers is in progress.

A 6-month extension to the project is requested to allow us sufficient time to fully test the potential of both the antibodies and the electronic transducers we have produced during the past 2½ years.