A New Phytochemical Screening Programme used for Crops grown with Organic and Conventional Methods

Screening process

Sample preparation
↓
Application on stationary phase
↓
Development in mobile phase
↓
Derivatization with chemical reagent
↓
Detection of phytochemical differences
↓
Evaluation and documentation of phytochemical differences

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Resumé

Et bredt screeningsprogram, til detektion af de mest generelle fytokemiske stoffer, blev udarbejdet ved hjælp af tyndtlagskromatografi (TLC). I alt blev 46 TLC systemer, bestående af 26 derivatiseringsreagenser, 3 stationære faser og 4 mobile faser, medtaget i screeningsprogrammet. TLC systemerne blev inddelt i grupper alt efter hvilke fytokemiske stoffer, de detekterede: alkoholer og fenoler, sukkerstoffer, N-holdige stoffer, organiske syrer og lipider, P-holdige stoffer, S-holdige stoffer og terpenoider. En sidste gruppe af TLC systemer detekterede stoffer fra flere af de ovennævnte grupper.

Kartofler (*S. tuberosum* L.), ærter (*P. sativum* L.), grønkål (*B. oleracea* L.), gulerødder (*D. carota* L.) og æbler (*M. domestica* Borkh.) dyrket med kombinationer af økologiske og konventionelle metoder til plantebeskyttelse og næringstilførsel, blev screenet for fytokemiske forskelle (biomarkører) ved hjælp af det udarbejdede screeningsprogram.

Karakteristiske fytokemiske forskelle blev fundet mellem afgrøder dyrket med forskellige metoder. I prøver fra ærter og gulerod blev der fundet én biomarkør. I ærter kunne biomarkøren relateres til jordbehandlingen, mens biomarkøren i gulerødder kunne relateres til brug af sprøjtemidler. I kartoffel blev der fundet to biomarkører relateret til brugen af sprøjtemidler. Tre biomarkører blev fundet i grønkål. To af disse kunne relateres til brugen af pesticider, mens den sidste kunne relateres til enten brug af kunstgødning eller jordbehandling. En række biomarkører blev fundet i æbler, men relationen til dyrkningsmetoderne var ikke umiddelbar klar. Tre af biomarkørerne i æbler kunne relateres til enten brugen af pesticider eller kunstgødning, mens der ikke kunne drages nogen konklusion for de øvrige.

Resultaterne fra screeningen af afgrøderne danner basis for udviklingen af en simpel og billig test til at detektere, hvorvidt afgrøder er økologisk eller konventionelt dyrkede. Endvidere danner resultaterne, sammen med andre resulter fra projektet "Økologisk kost og sundhed – et flergenerationers dyreforsøg", baggrund for udvælgelsen af, hvilke stoffer der skal kvantificeres ved kemisk analyse, isoleres og/eller strukturopklares.

Abstract

A broad screening programme, covering the most general phytochemical groups of compounds, was developed on the basis of Thin Layer Chromatography (TLC). A total of 46 TLC systems, comprising 26 derivatization reagents, 3 stationary phases, and 4 mobile phases, were included. The TLC systems were classified according to the groups of phytochemical compounds detected: Alcohols and phenolic compounds; Carbohydrates; N-containing compounds; Organic acids and lipids; P-containing compounds; S-containing compounds, and Terpenoids. Furthermore, one group of TLC systems detected compounds from several of the mentioned groups.

The screening programme was applied in the screening of potatoes (*S. tuberosum* L.), peas (*P. sativum* L.), kale (*B. oleracea* L.), carrots (*D. carota* L.), and apples (*M. domestica* Borkh.), cultivated with combinations of organic and conventional methods for plant protection and nutrient supply, for phytochemical differences (biomarkers).

Distinctive phytochemical differences were found between the differently cultivated samples of these crops. In peas and carrots only one biomarker was found. In peas the biomarker was related to the soil conditions, while the biomarker in carrots was related to the use of pesticides. In potato, two biomarkers related to the use of pesticides were found. Three biomarkers were found in kale. Two of these could be related to the use of pesticide, while the last was related to either fertiliser or soil conditions. Several biomarkers were found apples, but a relation to the cultivation methods was not clear. Three of the biomarkers in apples could be related to either the use of pesticides or fertiliser, while no conclusions could be drawn from the other biomarkers found.

The results of the screening programme form the basis for a potential development of a kit to detect whether crops are organically- or conventionally cultivated. Furthermore, the results from this part and other parts of the project “Organic food and health – a multigenerational animal experiment” provide basis for the selection of which secondary compounds to quantify by specific chemical analysis, isolate, and/or structure elucidation.

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1 Goal and introduction

1.1 Goal

The aim of this project is to,

- develop a screening programme covering the most general groups of phytochemical compounds using Thin Layer Chromatography as technique and
- perform a screening of crops grown under selected organic and conventional conditions (potatoes (*Solanum tuberosum* L.), kale (*Brassica oleracea* L.), carrots (*Daucus carota* L.), peas (*Pisum sativum* L.), and apples (*Malus domestica* Borkh.)) in order to detect possible phytochemical differences between organically and conventionally cultivated crops.

The results of the screening will form the basis for a potential development of a kit to determine whether crops are organically or conventionally cultivated. Furthermore, the results, from this part and other parts of the project "Organic food and health – a multigenerational animal experiment", will provide basis for the selection of which secondary compounds to quantify by specific chemical analysis, isolate, and/or structure elucidate.
A poster presenting the programme was presented at Natur- og Miljøforskningskonferencen in Copenhagen 22-23 August 2002.

1.2 Introduction

The introductory chapter presents the background for this project. The first section is about the effect on health of conventionally- and organically cultivated plant food. This section is followed by a description of a new assay method and kit for testing biological material for exposure to stress using biomarkers. The following section presents the project "Organic food and health – a multigenerational animal experiment" from which the plant material for this project was obtained. The fundamental differences between organic- and conventional cultivation methods are explained and the phytochemical differences previously detected between conventionally-

and organically cultivated farming food are then reviewed. This is succeeded by a short introduction to biomarkers and plant stress. In the literature several effect studies of crops exposed to different stress factors have been found. These studies are presented. The last section deals with Thin Layer Chromatography.

1.2.1 Conventional- versus organic farming and health

In the resent years there has been increasing focus on problems of food quality and considerable attention is being paid to organic farming.

Proponents of organic farming often claim that organically cultivated plant foods benefit more to health than conventionally cultivated plant foods. Others claim the opposite, and many doubt that there is any difference at all. The argument often used is that when plants are grown with fertilisers and pesticides, they are supposed to loose their natural defence mechanism. This is thought to result in reduced disease resistance and a diluted content of minerals, vitamins, and defence related secondary metabolites, of which the last are indiscriminately considered beneficial for human health. Opponents almost use the same argument to arrive at the opposite conclusion. Owing to inadequate nutrition of plants and lack of protection against diseases, organic products are supposed to contain less of protein, sugars, and vitamins and have increased levels of defence related secondary metabolites, which are in this case considered harmful for human health.

To solve this disagreement, knowledge about whether organically cultivated plant foods contain more or less of certain nutrients, minerals, vitamins, and secondary metabolites compared with conventionally cultivated plant foods, has to be obtained. Furthermore, it has to be investigated to what extent the above mentioned plant components are beneficial or harmful to health (Brandt & Mølgaard, 2001).

Many different investigations have shown that the greater the daily intake of vegetables and fruit, the smaller the risk of major deadly diseases as cancer (Gandini *et al*., 2000) and cardiovascular diseases (Ness & Powles, 1997). Vegetables and fruit are not major sources of vitamins, minerals, proteins, and carbohydrates in the average diet. In contrast, secondary metabolites are unique to these types of foods, and these compounds thus comprise the most likely candidates for this general health-promoting effect (Brandt & Mølgaard, 2001).

The use of pesticides implies a risk of accumulation of residues in conventionally cultivated plant foods which may lead to harmful effects. A study conducted by The National Danish Food Monitoring Program showed that in 1/3 of the conventionally cultivated fruit, pesticide residues were found, while only 5% of the conventionally produced vegetables contained pesticide residues. It was concluded that far from all conventionally grown fruits and vegetables contain pesticide residues and if they contain pesticides, the maximum residue limits are far from exceeded (Poulsen *et al*., 2000).

When plants are stressed from insect or fungal attack, they characteristically respond with a rapid increase in defence related secondary metabolites. Many of these compounds may cause mutagenicity, carcinogenity, teratogenity or neurotoxicity in laboratory tests. Since increases in toxic defence related secondary metabolites are stress mediated, the prevention of insect or fungal attack can reduce the levels of the toxic defence related secondary metabolites in our food supply. Pesticides used in conventional farming are used to reduce plant stress and there may be a possible benefit of decreased risk of toxic defence related secondary metabolites (Mattsson, 2000).
This project will only determine whether or not the phytochemical composition of organically and conventionally cultivated crops differs. To assess whether the differences are beneficial or harmful to health, the biological activity of the components must be determined, at the relevant concentrations.

1.2.2 Assay method and kit for testing biological material for exposure to stress using biomarkers

A new method for detection of biomarkers in biological material has been developed at the National Environmental Research Institute and sought patented internationally. The method is used to test whether a living organism has been exposed to stress, such as pesticides. The method is based on detection of a reproducible biomarker pattern, consisting of at least two biomarkers, in exposed living organisms compared with non-exposed living organisms. The biomarker pattern in living organisms exposed to stress is related to and depends on the applied stress. An object of the present invention is to provide an assay kit for the determination of whether material from a living organism has been exposed to stress. The assay kit is to be used as a field test or as laboratory test, in both cases with Thin Layer Chromatography as technique. An assay kit could be directly applicable in many fields. It could be used as an early warning system, where the phytochemical response to small amounts of herbicides appears before visual effects. Farmers could use the system to reduce the amount of pesticide necessary to obtain a given effect on plants. Furthermore, an assay kit could be used in food quality control, such as control of whether organic crops have been exposed to stress, such as pesticides (Ravn, 2001).

One of the studies providing basis for the above mentioned invention and patent, was a study including sixteen different wild plant species e.g. *Anagallis arvensis* L. and *Lolium perenne* L.,

representing nine families. The plants were exposed to four herbicides (metsulfuron methyl, glyphosate, pendimethalin and bromoxynil) with different modes of action and screened for phytochemical differences. A biomarker pattern was present in the most sensitive plant species, only 4 days after exposure with as low as 1% of recommended field dose of the herbicide, without visual effect on the exposed plants. A different biomarker pattern was present for each herbicide, and the biomarker pattern depended on the mode of action of the herbicide. Different species showed a different pattern of biomarkers, but a simpler common biomarker pattern was identified for all the species treated with the same herbicide. Furthermore, it was found that the different plant species had different sensitivity and that the response was delayed when plants were exposed to lower concentrations of herbicide. Besides, it was found that a biomarker pattern could be identified in a time period after exposure and until the death of the plant (Ravn & Løkke, 2002).
The phytochemical screening performed in this project will form an essential part in the process of developing a kit to determine whether crops are organically or conventionally cultivated.

1.2.3 The project "Organic food and health – a multigenerational animal experiment"

The overall objective of the project "Organic food and health – a multigenerational animal experiment" is to determine if a controlled animal feeding experiment comparing conventional and organic food products shows differences in animal physiology of a type and magnitude that indicates that such products can affect humans differently. The Danish Institute of Agricultural Science, The Royal Veterinary and Agricultural University, Risø National Laboratory, Technical University of Denmark, University of Southern Denmark and National Environmental Research Institute collaborate on the project.
Basically, the project consists of cultivation experiments. Seven crops (potato (*Solanum tuberosum* L.), pea (*Pisum sativum* L.), kale (*Brassica oleracea* L.), carrot (*Daucus carota* L.), apple (*Malus domestica* Borkh.), wheat (*Triticum aestivum* L.), and rape (*Brassica napus* L.)) are produced in three different models of cultivation systems,

- an organic cultivation system, without synthetic fertilisers and pesticides,
- a conventional cultivation system, using synthetic fertiliser and as much pesticides as allowed, and
- a factorial combination of the two other systems, with pesticides, but no synthetic fertiliser.

The picture-developing properties of the fresh plant material and feed mixtures are examined by the use of biocrystallization. Furthermore, characteristic secondary metabolites are measured in selected plant material from each cultivation system. The major nutrients in the plants are determined and the biological value of major protein sources of the plants will be assessed. Based on these results, 3 feed mixtures are prepared, corresponding to the 3 model systems. The concentration of approximately 20 elements and 150 pesticides are measured by analysis of the feed mixtures. Rats are fed with the feed mixtures and reproductive characteristics and weight gain is recorded. A screening for phytochemical differences in the plant material are performed. The data from the above mentioned experiments are assembled and analysed (FØJO, 2000, pp. 59-60).
The results of the screening for phytochemical differences are described in this report. These and results from other parts of the project will provide basis for selection of which secondary compounds to quantify by specific chemical analysis, isolate, and/or structure elucidate.

1.2.4 Organic- and conventional cultivation methods

In Denmark, the public interest in organic farming is fairly new and only few farms have followed the regulations for organic farming for more than a decade. The idea of organic farming goes back to the beginning of the 1900-century when fertiliserfertilisers first were introduced in agriculture. At that time, some farmers and scientists were concerned that this would lead to crops and soil of poor quality. Organic farming did not gain footing for many years since the benefits by using fertilisers and pesticides were obvious (Fog & Nørfelt, 2001, pp. 7). In the 1970s, attention was paid on the environmental impact of intensive agriculture, in particular problems with the use of pesticides and commercial fertilisers, was the major driving force (Thamsborg, 2001).
A fundamental principle in organic farming is to minimise environmental impacts as much as possible while sustaining an economically viable level of production. The key aspects of organic farming thus aim,

- to increase or at least maintain soil fertility over the long term,
- to avoid the use of mineral and synthetic fertilisers, and
- to avoid the use of synthetic pesticides.

The fertility of the soil may be defined as its ability to produce a satisfactory crop with minimal use of such resources as manure and fertilisers (Hansen *et al*., 2001). In terms of plant nutrition,

the main distinction between organic and conventional farming is the use of synthetic fertilisers in conventional farming as compared to only animal manure and crop residues in organic farming. The supply of nutrients is one of the most limiting factors in organic farming. Nitrogen self sufficiency is secured through the use of legumes crops which are capable of obtaining nitrogen directly from the air through bacterial nodules on their roots. Furthermore, nitrogen supply is secured through an effective recycling of organic materials, including crop residues and animal manure (Thamsborg, 2001). Microorganisms (bacteria and fungi) play a central role in maintaining the fertility of the soil, and are therefore crucial to organic farming. Within the soil, the decomposition of organic matter, releasing plant available N, is primarily due to microbial activity. Earthworms are key species of the macro-fauna, and are very important for soil fertility, being the first link in the decomposition of plant constituents. With regard to soil biology, due to its versatile crop rotations, reduced applications of nutrients, and the ban on pesticides, organic farming is usually associated with a significantly higher level of biological activity (Hansen *et al*, 2001).

Since the use of synthetic pesticides is banned in organic farming, weed, diseases and pests are kept under control by rotating crops to change the field ecology. Weeds are controlled through crop rotation, mechanical tillage, and hand weeding, as well as through flame weeding and other management methods (Fog & Nørfelt, 2001).

Synthetic pesticides, used in conventional farming, are deliberately used for the purpose of killing different forms of life. The ideal situation, of course, is that pesticides are highly selective, destroying target organisms while leaving non-target organisms unharmed (Hodgson & Levi, 1987, pp. 66). Pesticides are a general term, which includes chemicals used to control insects (insecticides), weeds (herbicides), plant diseases (fungicides) etc. Herbicides are substances used to eliminate unwanted plants in agriculture (Duffus, 1980, pp. 58). Herbicides represent an extremely broad array of chemical classes and, in turn, act at a large number of sites of metabolic function and energy transfer in plant cells. Despite a growing body of knowledge, the exact molecular sites of action of many herbicides are unknown (Duke, 1990).

Below, herbicides used in the cultivation experiments of crops ("Organic food and health – a multigenerational animal experiment") are described since they might have an influence on non-target plants. Insecticides and fungicides used in the cultivation experiments are not described here, since they are designed to kill fungi and insects, respectively, they do not have plants as their target, and no data is available on their effects on plants, if any.

Metribuzin is a selective systemic herbicide used in the control of many annual broad-leaved and grass weeds in e.g. potatoes (Tomlin, 2001). The selectivity in potatoes is the result of partly low absorption, due to treatment with metribuzin before germination of the potatoes, partly inactivation of metribuzin in the potatoes (Kristensen *et al*., 2000).
Bentazone is a selective contact herbicide used in the control of many annual broad-leaved weeds in e.g. peas (Tomlin, 2001).
Linuron is a selective systemic herbicide. Linuron is used in the pre- and post-emergence control of annual grass and broad-leaved weeds, and some seedling perennial weeds in e.g. carrots (Tomlin, 2001). The selectivity in carrots is a result of inactivation of linuron in the roots (Kristensen *et al*., 2000).
The above mentioned herbicides all act as photosynthetic inhibitors. They all bind to the D-1, quinone-binding protein of photosynthetic electron transport and thereby block photosynthetic electron transport (Duke, 1990).
Glufosinate-ammonium is a non-selective contact herbicide with some systemic action, used as a desiccant in e.g. potatoes. The herbicide is an irreversible inhibitor that competitively inhibits binding of glutamate to glutamine synthetase (GS). The inhibition of GS in plants that are reducing nitrate to ammonia leads to accumulation of toxic levels of ammonia and rapid cellular collapse (Duke, 1990).
Pendimethalin is a selective herbicide used in the control of most annual grasses and many annual broad-leaved weeds in e.g. peas (Tomlin, 2001). The herbicide directly disrupts cell division by attacking a molecular site that is specific for cell division. The herbicide binds tubulin, the protein from which microtubules are composed. Microtubules are required for cell division and cell wall formation (Duke, 1990).
In this project, potential phytochemical differences between organically and conventionally cultivated crops will, when possible, be related to either soil conditions, fertiliser or to the use of pesticides. The mode of action of the herbicides used will be included in the discussion of the phytochemical differences related to pesticides.

1.2.5 Phytochemical differences between organically and conventionally cultivated food

Two recent reviews (Woese *et al*., 1997 and Williams, 2002) covering more than 150 comparative studies of organically and conventionally cultivated plant food e.g. vegetables and fruit, concluded that there were only small and inconsistent differences. Only for nitrate and vitamin C (ascorbic acid) systematic tendencies were apparent. Lower nitrate levels, resulting in

fewer N-containing compounds, and higher vitamin C (ascorbic acid) levels were found in organically cultivated plant food compared with conventionally cultivated plant food. An experiment was conducted for three years with five replicates of two treatments, one organic and one conventional, for potatoes (*S. tuberosum* L.)(Warman & Havard, 1998), carrots (*D. carota* L.), and kale (*B. oleracea* L.)(Warman & Havard, 1997). Given the number of factors analysed for carrots, kale, and potatoes, there were relatively few and small differences in the yield and in the vitamin and mineral content using the two different cultivation systems. The study found that the vitamin C (ascorbic acid) level in potatoes, carrots, and kale was not affected by the different cultivation methods.

In most studies, only nutrients, vitamins, and minerals were analysed. The secondary metabolites, which are the compounds, which both pro and cons expect to differ between organically and conventionally cultivated plant food, were not analysed.

In this project, a broad range of phytochemical compounds organically will be investigated in crops grown in three models of organic and conventional cultivation systems. Screening for N-containing compounds and organic acids will indicate whether some of the above-described relations apply to the crops tested.

1.2.6 Biomarkers and plant stress

A biomarker is defined as: "A biological response to an environmental chemical which gives a measure of exposure, and sometimes also of toxic effect. The biological response may be at the molecular, cellular or whole organism level" (Walker, 1995).

Plant stress refers to a wide range of biological, chemical, and physical stress that crops and other plants are subjected to (Lichtenthaler, 1996). Stress factors can be divided into natural- and anthropogenic stress factors. Some of the different kinds of stress factors acting on plants and crops are listed in Figure 1 under the grouping of natural- and anthropogenic stress factors. The various stress factors can also be listed under biotic and abiotic stress factors which is as valid as the grouping in Figure 1 (Lichtenthaler, 1996).

Figure 1: List of some different natural- and anthropogenic stress factors.

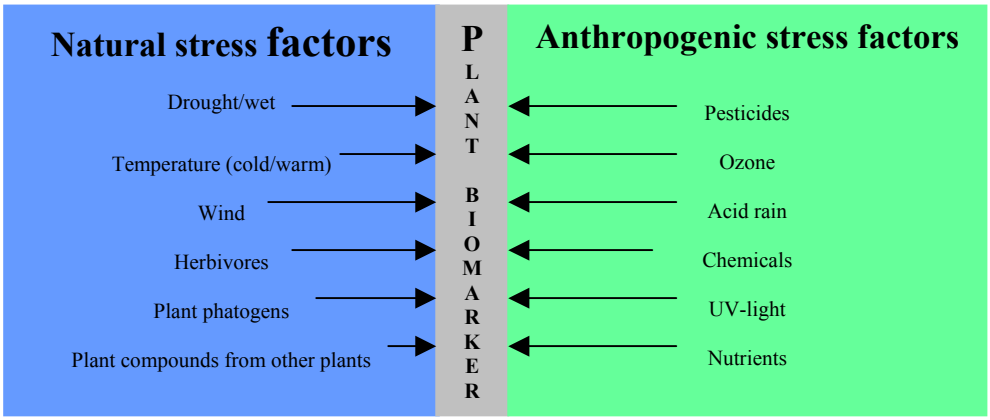


Figure modified according to Lichtenthaler (1996).

Some of the anthropogenic stress factors mentioned in Figure 1, UV-light, nutrients, and ozone, can also be considered as natural stress factors.

In this project, a biomarker refers to a phytochemical compound that increases or decreases in concentration as a response to an exposure to anthropogenic chemical stress. The phytochemical compound increases or decreases at least 50% in concentration, in relation to a control, in both replicates, before it is considered a biomarker. A biomarker pattern is the overall phytochemical differences assembled and consists of at least 2 biomarkers.
The anthropogenic chemical stress factors in this project include pesticides and fertilisers.

1.2.7 Effect studies of crops exposed to plant stress

A literature study has been performed in order to illustrate different aspects of stress exposure in crops. Different natural- and anthropogenic stress factors, as well as different crops, relevant to this project, have been included.

The following two examples illustrate the influence of climatic conditions (Rosa *et al*., 2001) and turnip root fly damage (Hopkins *et al*., 1995) on the level of carbohydrates in kale (*B. oleracea* L.).
The first study investigated the influence of climatic conditions on glucose, fructose, and sucrose levels in kale (*B. oleracea* L.) grown in spring/summer and summer/winter. The kale was grown in the same field and harvested at commercial maturity stage. The leaves were freeze dried and extracted with ethanol before the carbohydrates were measured spectrophotometrically. The

level of glucose was higher in spring/summer than in summer/winter season while the exact opposite was found for fructose levels. Only a slight increase in sucrose was seen in spring/summer compared to summer/winter (Rosa *et al*., 2001).
The content of fructose, glucose, sucrose, and total sugars was measured in kale (*B. oleracea* L.) inoculated with eggs of the turnip root fly, *Delia floralis*. Freeze-dried root material was extracted with ethanol and the carbohydrates were separated and quantified by HPLC (High Performance Liquid Chromatography). The fructose concentration rose while the glucose, sucrose, and total sugar concentration fell after inoculation (Hopkins *et al*., 1995). The decrease in total root carbohydrate concentration may be explained in two ways. Carbohydrate production may be limited by the root damage caused by larval feeding. Secondly, it is possible that the parts of the root higher in carbohydrate concentration will be consumed first, resulting in a decrease in total root carbohydrate concentration.
The two above-mentioned examples indicate that the carbohydrate level in kale changes in response to several stress factors.

Examples covering drought stress in the literature are numerous. The following example illustrates how different varieties of the same species respond phytochemically different to the same stress factor.
Three different varieties of *S. tuberosum* L. grown under drought stress, with and without irrigation, were analysed for their content of glycoalkaloids. The whole potato was freeze dried, extracted and quantified by the use of HPLC. A significant increase in the concentration of glycoalkaloids was observed under drought stress conditions in *S. tuberosum* var. *andigena* (Malcacho) and *S. tuberosum* var. *tuberosum* (Desiree) while only a small increase in the concentration of glycoalkaloids was observed in *S. tuberosum* var. *andigena* (Sani Imilla) (Bejarano *et al*., 2000). Since the potatoes were freeze dried the differences seen in the glycoalkaloid content cannot be explained in terms of varying water content in the potatoes. Therefore, the conclusion must be that different varieties react differently upon the same stress factor, *S. tuberosum* var. *andigena* (Sani Imilla) being the most tolerant to drought stress.

Anthocyanins are, due to their UV radiation absorbing characteristics, considered as protective agents against harmful effects of UV radiation. Anthocyanins also function as pigments.

In a study peformed by Merzlyak & Chivkunova (2000), it was investigated if anthocyanins were also involved in the defence against visible radiation. The amount of anthocyanins in fruits of apples (*M. domestica* Borkh.) grown in sunlight and shade was measured using reflectance spectroscopy. Information on how the plant material was grown, and how the samples were prepared is missing. The results showed that apples grown in sunlight accumulated large amounts of anthocyanins while apples grown in shade did not accumulate anthocyanins. Furthermore, the sunlit side of the apples was pink or dark red while the shaded side was pale green. The change of apple colour to dark red was accompanied by a rise of cyanidin-3-galactosid, which was the main anthocyanin in the apple skin.

The aspect of inter-relationships between two different stress factors is covered by investigations performed by Alexieva *et al*. (2001). The stress responses and interaction of drought and ultraviolet-B radiation in peas (*P. sativum* L.) were examined.

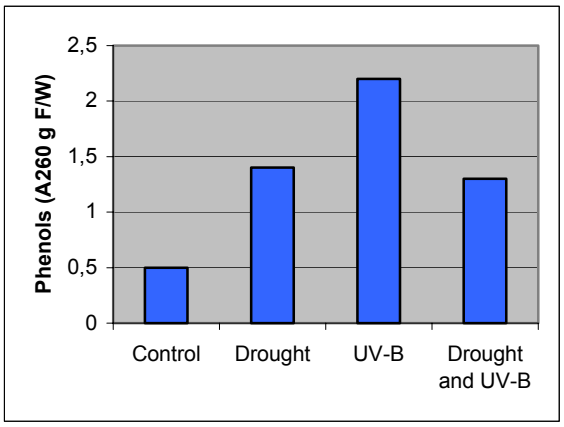


Figure 2: Changes in the content of phenols in peas (*P. sativum* L.) subjected to drought and UV-B stresses (Alexieva *et al*., 2001).

The peas were in the age of 10 days exposed to drought stress for 7 days. The plants were harvested and the leaves together with water were shaken for 24 hours. The amount of phenols was measured spectrophotometrically.
Among the results was found that the amount of phenols was affected by the application of drought and UV-B radiation stress in pea, see Figure 2. A stronger increase in the concentration of phenols was observed after application of UV-B radiation compared to the application of drought stress. Furthermore, the two stress factors acted synergistically on the amount of phenols

to induce protective mechanisms in that pre-application of either stress reduced the damage caused by subsequent application of the other stress.
The protective interaction between UV-B radiation and drought stresses may be that the control of water loss constitutes an UV-B radiation positive effect on drought stressed pea plants.
Another experiment investigated the effects of water-deficit stress on the content of carbon and nitrogen in *P. sativum* L. nodules. 4-week-old nodules were exposed to mild and intense water-deficit stress for 7 and 14 days respectively. Information on sample preparation and on how the carbon and nitrogen content was measured is missing. A drastic increase in the nodules content of sucrose was found while glucose and fructose did not show any response to any of the imposed water-deficits. Nodule total free amino acids increased throughout the mild and intense water stress period. Amino acid levels fell to control values at the end of the intense water stress period (Gonzáles *et al*., 1998). The ability to metabolise sucrose may be impaired in water-deficit stressed pea nodules. The increase in amino acid level may be due to less water for the transport of N-products away from the nodules. This, however, does not explain why the amino acid level fell to control values at the end of the intense water stress period.

The effects of mechanical stress on the levels of certain carbohydrates and terpenes in different varieties of carrots (*D. carota* L.) were investigated. Hand harvested carrots were exposed to mechanical stress by shaking in a shipping stress simulator. The carrots were freeze dried, extracted and quantification of terpenes and carbohydrates was done by the use of HPLC. The phytochemical differences seen as a response to mechanical stress differed among the varieties. The content of bornyl acetate, β-pinene and total terpenes was lower in the mechanically stressed carrots. Furthermore, a reduced content of sucrose and glucose was found in the mechanically stressed carrots. Mechanical stress did not affect the content of fructose. An increase in respiration was also found in the mechanically stressed carrots (Seljåsen *et al*., 2001). The increase in respiration is expected to result in an increase in the utilisation of energy reserves explaining the decrease in sucrose and glucose content. Mechanical wounding of the carrots, caused by shaking, may have led to the loss of volatile substances as volatile terpenes, explaining the decrease in content of individual terpenes and total terpenes.

Only one or two phytochemical groups of compounds have been described, in response to a given stress factor, in the above-mentioned studies. In this project, a broad range of phytochemical compounds will be investigated.

1.2.8 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a relatively new discipline. Chromatography historians usually date the advent of modern TLC to 1958. Early development in TLC was done by Izmailov and Schraiber in 1938. They succeeded in separating medicinal plants on unbound alumina spread on glass plates. They applied drops of solvents to the plate containing the sorbent layer and sample, as a result several circles of substances were seen in UV-light. In 1949 Meinhard and Hall used a binder to adhere alumina to microscope slides. These layers were used in the separation of inorganic ions. In the early 1950s Kirchner developed TLC as we know it today. He was the first to separate mixtures by adsorption chromatography on filter paper and, later, on glass fibre paper impregnated with silica and alumina. Stahl introduced the term "Thin Layer Chromatography" in the late 1950s. His major contributions were standardisation of materials, procedures, and description of selective solvent systems for resolution of important compound classes (Sherma & Fried, 1996, pp. 4-5; Kreuzig, 1998; Scott, 1995, pp. 349-351).

Figure 3: TLC equipment.

TLC can either be a manual procedure or it can be partly or completely automated.
At the Department of Terrestrial Ecology, a complete CAMAG TLC equipment, supported by The Danish Agricultural and Veterinary Research Council has been acquired. The equipment consists of a fully automatic sample applicator, an automatic developing chamber, and an automatic immersion (dipping) devise. Another part of the equipment is an image documentation system, VideoStore, which allows imaging and archiving of chromatograms while VideoScan allows evaluation of the images captured with VideoStore. Finally, a scanner is available allowing densitometric evaluation of chromatograms.
Modern TLC is an extremely simple, flexible, reliable, and cost efficient method (Fried & Sherma, 1999, pp. 5). It can be used for separation, isolation, identification, and quantification of sample components (Fried & Sherma, 1999, pp. 1). Thin Layer Chromatography is widely used

in many different fields e.g. medicine, pharmacy, environment, food, and chemistry (Hahn-Deinstrop, 2000, pp. 3).

TLC is the technique used in this project. The described equipment will be applied in the screening of crops.

2 Experimental

2.1 Apparatus

Dip tanks (Duran glass) for plates 20 x 20 cm (22.6629), CAMAG, Muttenz, Switzerland

Lid for dip tanks 20 x 20 cm (22.6622), CAMAG, Muttenz, Switzerland

Disposable Micropipettes (2 and 5 µl) and pipetting aids, BLAUBRAND ® intraEND, Brand

Elgastat Maxima Analytical, Holm & Halby, Allerød, Denmark

Flat Bottom Chamber for 10 x 10 cm plates, with stainless steel lid (022.5150), CAMAG, Muttenz, Switzerland

Hand press, SUSI, Zyliss, U.S.Pat. 5.513.562, Switzerland

Micro Centrifuger, Capsule, 6.400 rpm/min, Tomy Seiko CO. Ltd., Tokyo, Japan

Moulinex LUXE Coffee Mill, type 843

Plastic Bottles, polyethylene, 100 ml, Apodan, Copenhagen, Denmark

Screw caps with dosing hole and protective lids, Apodan, Copenhagen, Denmark

Reagent Spray (atomizer) and spray head, CAMAG, Muttenz, Switzerland

Refrigerating centrifuger type 154.RF, Ole Dich Instrumentmakers ApS, Hvidovre, Denmark

Reprostar 3 with cabinet cover, mounted digital camera, and camera UV blocking filter typ. 2A, CAMAG, Muttenz, Switzerland

TLC Plate Heater III, CAMAG, Muttenz, Switzerland

TLC Spray Cabinet, CAMAG, Muttenz, Switzerland

Ultrasonic Bath, Branson 5510E-MT, Bransonic®, Danbury, USA

UV Lamp dual wavelength, 254/366 nm in combination with Viewing Box 3, CAMAG, Muttenz, Switzerland

VideoScan, Version 1.01, (22.9579), CAMAG, Muttenz, Switzerland

VideoStore 2, Version 3.00, (22.9566), CAMAG, Muttenz, Switzerland

VitamiX Saftcentrifuge, OBH, Ole Bøtcher-Hansen A/S

2.2 Materials

The materials listed in this part of the report are included in the screening programme. Materials only used in the development of the screening programme are presented in Appendix 1.

2.2.1 TLC plates

Silica gel 60, HPTLC aluminium sheets 20 x 20 cm, Merck Ord. No. 1.05547

Silica gel 60 F_{254}, HPTLC aluminium sheets 20 x 20 cm, Merck Ord. No. 1.05548

Cellulose, TLC aluminium sheets 20 x 20 cm, Merck Ord. No. 1.05552

2.2.2 Reagents

Acetic acid 100%, GR for analysis, ACS, ISO, Merck Ord. No. 1.00063

o-Anisaldehyde 98%, Acros Organics Ord. No. 14922

Ammonia solution 24,5%, Borup Kemi

Ammonia solution 25%, GR for analysis, Merck Ord. No. 1.05432

Ammonium molybdate, GR for analysis, ACS, ISO, Merck Ord. No. 1.01182

Bismuth(III)nitrate, BDH Laboratory Supplies Prod. No. 27388

Bromocresol green, indicator pH 3.8-5.4, Fluka® Prod. No. 17470

Bromophenol blue, indicator pH 3.0-4.6, ACS, Merck Ord. No. 1.08122

1-Butanol, GR for analysis, ACS, ISO, Merck Ord. No. 1.01990

Cobalt(II)chloride hexahydrate, GR for analysis, ACS, Merck Ord. No. 1.02539

Copper(II)nitrate, GR for analysis, Merck Ord. No. 1.02753

2',7'-Dichlorofluorescein, fluorescence indicator, Merck Ord. No. 1.09676

Diphenylboric acid 2-aminoethylester, Sigma® D 9754

Ethanol 96%, Ph. Eur., Danisco Distillers

Ethanol 99,9%, Ph. Eur., Danisco Distillers

Fluorescein, Aldrich® Prod. No. F245-6

Formic acid 98-100%, GR for analysis, ACS, Merck Ord. No. 1.00264

Hydrochloric acid 25%, GR for analysis, Merck Ord. No. 1.00316

Hydrochloric acid min. 37%, Analytical Reagent, Reag. ISO, Reag. Ph. Eur., Riedel-deHaën® Prod. No. 30721

Iodine resublimed, GR for analysis, ACS, ISO, Merck Ord. No. 1.04761

Iron(III)chloride anhydrous for synthesis, Merck Ord. No. 8.03945

Methanol, GR for analysis, ACS, ISO, Merck Ord. No. 1.06009

Methylene blue, Reag. Ph. Eur., Merck Ord. No. 1.59270

Molybdatophosphoric acid, GR for analysis, ACS, Merck Ord. No. 1.00532

β-Naphthol, Sigma® N 1250

Naphthoresorcinol, Fluka® Prod. No. 70650

Ninhydrin, GR for analysis, Merck Ord. No. 1.06762

Nitric acid 65%, GR for analysis, ISO, Merck Ord. No. 1.00456

Palladium(II)chloride (59%Pd) anhydrous, for syntesis, Merck Ord. No. 8.07110

Phosphoric acid 85%, "Baker Analyzed", ACS, J.T.Baker® Prod. No. 6024

Polyethylene glycol 4000 for synthesis, Merck Ord. No. 8.07490

Potassium iodide, GR for analysis, ISO, Merck Ord. No. 1.05043

Potassium permanganate "Baker Analyzed", ACS, J.T.Baker® Prod. No. 0237

1-Propanol, GR for analysis, Merck Ord. No. 1.00997

2-Propanol, GR for analysis, ACS, ISO, Merck Ord. No. 1.09634

Rhodamine 6 G, Sigma® R 4127

Rhodamine B, Sigma® R 6626

Silver nitrate, GR for analysis, ACS, ISO, Merck Ord. No. 1.01512

Sodium carbonate anhydrous, GR for analysis, ISO, Merck Ord. No. 1.06392

Sodium nitrite, GR for analysis, ACS, Merck Ord. No. 1.06549

Sulfuric acid 95-97%, GR for analysis, ISO, Merck Ord. No. 1.00731

Thymol, Fluka® Prod. No. 89330

Tin(II)chloride, GR for analysis, ACS, Merck Ord. No. 1.07815

Vanillin, Ph. Eur., BP, NF, Merck Ord. No. 1.08510

Water, ELGA, ion exchanged water (Elgastat Maxima Analytical)

Zinc chloride, GR for analysis, ACS, ISO, Merck Ord. No. 1.08816

2.2.3 Plant material

Plant material included in this project:

- Potato, *Solanum tuberosum* L. (Sava) (Solanaceae)
- Pea, *Pisum sativum* L. (Ambassador) (Fabaceae)
- Kale, *Brassica oleracea* L. (Bona) (Brassicaceae)
- Carrot, *Daucus carota* L. (Bolero) (Apiaceae)

- Apple, *Malus domestica* Borkh. (Otava) (Malaceae)

Potatoes (*S. tuberosum* L.) were produced in Foulum. Carrots (*D. carota* L.), kale (*B. oleracea* L.), apples (*M. domestica* Borkh.), and peas (*P. sativum* L.) were produced in Årslev. The plots with pesticide treated crops and untreated crops respectively were placed in the same or immediately adjacent fields to ensure comparable soil conditions. In all cases the pesticide treated plots were placed with concern for the risk of contamination, an appropriate separation was defined between the plots, and spraying was done with appropriate equipment, only under suitable weather conditions. The cultivation methods for the individual crops are shown in Table 1-5 below.

Table 1: Cultivation method, potato.

Crop: *Solanum tuberosum* L., **Cultivation locality:** Foulum, **Time of planting:** 24/4-2001, **Time of harvesting:** 27-28/9-2001, **Storage temperature:** 6°C

	Parcel 1 " Pseudo conventional"[3]		**Parcel 2**		**Parcel 3 "Organic"**
Soil	OrganicOrganic[2]		OrganicOrganic[2]		OrganicOrganic[2]
Fertiliser	High (NPK, 135 kg N/ha, 19 kg P/ha, 65 kg K/ha)		Low (Animal manure, 63 kg N/ha, 24 kg P/ha, 40 kg K/ha)		
Pesticide	Pesticide				No pesticide
	Date	**Pesticide - active ingredient**	**Kg. pr. ha.**	**Type of pesticide**	
	-[1] 3/7-2001 -[1] -[1]	Metribuzin Mancozeb Cypermethrin Glufosinat-ammonium	0.35 2 0.2 3	Herbicide Fungicide Insecticide Herbicide	

[1] Information about date not available; [2] Soil cultivated organically for at least 5 years; [3] No real conventional cultivation method since organicorganic soil is used.

Table 2: Cultivation method, pea.

Crop: *Pisum sativum* L., **Cultivation locality:** Årslev, **Time of sowing:** 2/5-2001, **Time of harvesting:** 23/8-2001 (sundried), **Storage temperature:** -18°C

	Parcel 1 "Conventional"		**Parcel 2**		**Parcel 3 "Organic"**
Soil	Conventional[1]		OrganicOrganic[2]		OrganicOrganic[2]
Pesticide	Pesticide				No pesticide
	Date	**Pesticide - active ingredient**	**Kg. pr. ha.**	**Type of pesticide**	
	16/5-2001 21/5-2001 26/6-2001 11/7-2001 12/7-2001	α-cypermethrin α-cypermethrin Pendimethalin Bentazone α-cypermethrin Mancozeb Pirimicarb	0.2 0.2 1.5 1 0.2 2 0.5	Insecticide Insecticide Herbicide Herbicide Insecticide Fungicide Insecticide	

[1] Soil cultivated conventionally for at least 5 years; [2] Soil cultivated organically for at least 5 years.

Table 3: Cultivation method, kale.

Crop: *Brassica oleracea* L., **Cultivation locality:** Årslev, **Time of sowing:** 3/5-2001, **Time of planting out:** 30/5-2001, **Time of harvesting:** 30/10-2001, **Storage temperature:** -18°C

	Parcel 1	**Parcel 2 "Conventional"**	**Parcel 3**	**Parcel 4 "Organic"**
Soil	Conventional[5]	Conventional[5]	OrganicOrganic[6]	OrganicOrganic[6]
Fertiliser	Low (NPK[1])	High (NPK[1])	Low (Binadan[2])	Low (Binadan[2])
Pesticide	Pesticide			No pesticide

Date	**Pesticide - active ingredient**	**Kg. pr. ha.**	**Type of pesticide**
-[3]	Thiram	Steeping of seeds	Fungicide
28/5-2001	Chlorfenvinphos	-[4]	Insecticide
13/6-2001	Dimethoate	2	Insecticide
13/7-2001	Pirimicarb	0.5	Insecticide
16/7-2001	Biobit	1	Insecticide
26/7-2001	Biobit	1	Insecticide
10/8-2001	Biobit	1	Insecticide
15/8-2001	Mancozeb	2	Fungicide
27/8-2001	Pirimicarb	0.5	Insecticide
29/8-2001	Biobit	1	Insecticide
11/9-2001	Mancozeb	2	Fungicide

[1]Information about the composition of NPK not available; [2] Organic pellets of poultry manure; [3] Information about date not available; [4] Information about amount of chlorfenvinphos not available; [5] Soil cultivated conventionally for at least 5 years; [6] Soil cultivated organically for at least 5 years.

Table 4: Cultivation method, carrot.

Crop: *Daucus carota* L., **Cultivation locality:** Årslev, **Time of sowing:** 23/5-2001, **Time of harvesting:** 17/10-2001, **Storage temperature:** -18°C

	Parcel 1 "Conventional"	**Parcel 2**	**Parcel 3 "Organic"**
Soil	Conventional[4]	OrganicOrganic[5]	OrganicOrganic[5]
Fertiliser	High (NPK[1])	Low (released from last years crop[2])	Low (released from last years crop[2])
Pesticide	Pesticide		No pesticide

Date	**Pesticide - active ingredient**	**Kg. pr. ha.**	**Type of pesticide**
-[3]	Iprodione, Thiram & Metalaxyl	Steeping of seeds	Fungicides
28/6-2001	Linuron	1	Herbicide
6/7-2001	Linuron	1	Herbicide
17/8-2001	α-cypermethrin	0.2	Insecticide
21/8-2001	α-cypermethrin	0.1	Insecticide
11/9-2001	Iprodione	1.2	Fungicide

[1] Information about the composition of NPK not available; [2] Information about last years crop not available; [3] Information about date not available; [4] Soil cultivated conventionally for at least 5 years; [5] Soil cultivated organically for at least 5 years.

Table 5: Cultivation method, apple.

Crop: *Malus domestica* Borkh., **Cultivation locality:** Årslev, **Time of harvesting:** Primo October 2001, **Storage temperature:** -18°C

	Parcel 1	**Parcel 2 "Organic"**	**Parcel 3 "Pseudo conventional"**[3]		**Parcel 4**	
Soil	OrganicOrganic[2]	OrganicOrganic[2]	OrganicOrganic[2]		OrganicOrganic[2]	
Fertiliser	High N (*T. resupinatum* L. and *L. multiflorum* Lam.)	Low N (Grass[1])	High N (*T. resupinatum* L. and *L. multiflorum* Lam.)		Low N (Grass[1])	
Pesticide	No pesticide		Pesticide			
			Date	**Pesticide - active ingredient**	**Kg. pr. ha.**	**Type of pesticide**
			23/5-2001	Kresoximmethyl	0.2	Fungicide
			31/5-2001	Kresoximmethyl	0.2	Fungicide
			6/6-2001	Dithianon	1.0	Fungicide
				Phosalon	1.5	Insectcide
			15/6-2001	Mancozeb	3.0	Fungicide
			22/6-2001	Dithianon	1.0	Fungicide
			29/6-2001	Triforin	2.5	Fungicide
			9/7-2001	Dithianon	1.0	Fungicide
				Calcium nitrate	6.0	-
			20/7-2001	Dithianon	1.0	Fungicide
				Calcium nitrate	6.0	-
			2/8-2001	Dithianon	1.0	Fungicide

[1] Information about the composition of grass not available; [2] Soil cultivated organically for at least 5 years; [3] No real conventional cultivation since organicorganic soil is applied.

2.3 Method

2.3.1 Sample preparation

2.3.1.1 Potato (S. tuberosum L.)

Five fresh potatoes were rinsed with water and separated into peel and core respectively. Juice from peel and core respectively was prepared in a juice separator. The juice was transferred to micro tubes and centrifuged (6.400 rpm) for 3 minutes. The supernatant was centrifuged for additionally 3 minutes in new micro tubes. The supernatant was immediately used for phytochemical analysis.

2.3.1.2 Pea (*P. sativum* L.)

Twenty frozen mature dry peas were defrosted and crushed in a coffee mill. 250 mg was extracted with 2.50 ml 75% ethanol in an ultrasonic bath for 2 hours. Ice was added to the bath every 30 minutes to avoid decomposition of the sample components. The extract was transferred to micro tubes and centrifuged (6.400 rpm) for 3 minutes. The supernatant was centrifuged for additionally 3 minutes in new micro tubes. The supernatant was used for phytochemical analysis and was kept cool (2°C) until the analysis was performed the following day.

2.3.1.3 Kale (*B. oleracea* L.)

10 g defrosted kale was separated from stems and pressed for plant sap using a hand press. The first ml was discarded. The remaining sap was transferred to micro tubes and centrifuged (6.400 rpm) for 3 minutes. The supernatant was centrifuged for additionally 3 minutes in new micro tubes. The supernatant was immediately used for phytochemical analysis.

2.3.1.4 Carrot (*D. carota* L.)

Three frozen carrots were defrosted and rinsed with water. The first cm of the top of the root was discarded and of the remaining only the 5 cm top of the root was used. This was performed to obtain a homogeneous root tissue. Juice was prepared in a juice separator. The juice was transferred to micro tubes and centrifuged (6.400 rpm) for 3 minutes. The supernatant was centrifuged for additionally 3 minutes in new micro tubes. The supernatant was immediately used for phytochemical analysis.

2.3.1.5 Apple (*M. domestica* Borkh.)

Five frozen apples were defrosted and freed from most of the core only leaving about 1 cm of the core together with the peel. Juice was prepared in a juice separator. The juice was transferred to micro tubes and centrifuged (64.000 rpm) for 10 minutes. The supernatant was centrifuged for additionally 10 minutes in new micro tubes. The supernatant was immediately used for phytochemical analysis.

2.3.2 Screening procedure

The screening programme, presented in Table 6, was run for each crop. Replicate experiments were carried out for each crop with different representative samples to ensure reliability of the results.

The development of the screening programme is presented in section 3.1.

Table 6: Screening programme. TLC systems classified according to the group of phytochemical compounds detected.

Alcohols and phenolic compounds	Carbohydrates	N-containing compounds	Organic acids and lipids	P-containing compounds	S-containing compounds	Terpenoids	Several of the mentioned groups
V (52,8)	AA (47,1) AA (47,8) AD_{β} (47,8) AI (47,8)	AL (48,8) C (47,8) C (52,2) C (52,8) E (47,1) E (47,8)	AN (47,1) AN (47,8) AN (52,1) AV (47,8) AX (52,1) AX (52,2) AX (52,3) AY (52,8) G (47,1) G (47,8) G (52,8) N (52,1) N (52,8)	BH (47,1) BH (47,8) BI (47,8)	AÅ (47,1) AÅ (47,8) AÅ (52,1) BG (52,1) BG (52,8)	BB (47,1) BB (47,8) YB (47,8) Z (47,8) Z (52,8)	A (47;8) B (47,1) B (47,8) BC (47,1) F (47,1) F (47,8) I (47,1) I (47,8) R (47,8)

Capital letter: Indicates the derivatisation reagent, see Table 16; **Numbers in brackets**: Indicate the stationary- and mobile phase; **47**: Silica gel 60, HPTLC aluminium sheets; **48**: Silica gel 60 F_{254}, HPTLC aluminium sheets; **52**: Cellulose, TLC aluminium sheets; **1**: 1-butanol:acetic acid:water (4:1:5), upper phase; **2**: 1-butanol:50% formic acid (2:1); **3**: 2-propanol:acetic acid (2:1); **8**: 1-propanol:25% ammonia (11:9).

2.3.3 Preparation of TLC plates

The TLC plates used for the screening were 6.67 x 10 cm plates for carrot (*D. carota* L.) and pea (*P. sativum* L.) and 10 x 10 cm plates for potato (*S. tuberosum* L.), apple (*M. domestica* Borkh.), and kale (*B. oleracea* L.). The starting line was marked at a distance of 1.5 cm from the bottom edge of the plate and the distance to be reached by the solvent front was marked at a distance of 9.5 cm from the bottom edge of the plate. The marks were made with a soft pencil.

2.3.4 Application on TLC plates

The extracts were applied pointwise using 2 and 5 µl micropipettes. Extracts of potato (*S. tuberosum* L.), apple (*M. domestica* Borkh.), carrot (*D. carota* L.), and kale (*B. oleracea* L.) were applied in the amounts of 5 µl on Silica gel 60, HPTLC aluminium sheets and Silica gel 60 F_{254}, HPTLC aluminium sheets and 2 µl on Cellulose, TLC aluminium sheets. Silica gel 60, HPTLC aluminium sheets used in combination with the following derivatisation reagents: R (silver nitrate - ammonia), AI (thymol - sulfuric acid), AD_{β} (β-naphthol - sulfuric acid), and AA (naphthoresorcinol - sulfuric acid) were applied the amount of 2 µl of extract. Extracts from pea (*P. sativum* L.) were applied in the amount of 5 µl on all plate types.

2.3.5 Preparation of mobile phases

Four different mobile phases were employed in the screening programme:

Mobile phase no. 1: 1-butanol:acetic acid:water (4:1:5).

The mobile phase was prepared by mixing 1-butanol, acetic acid and water in the proportion of 4 to 1 to 5 in a separatory funnel and shaking manually for 10 minutes. The two phases separated and the upper phase was used as mobile phase. Previous stability studies showed that the mobile phase was stable for minimum 7 days.

Mobile phase no. 2: 1-butanol:50% formic acid (2:1).

Mobile phase no. 3: 2-propanol:acetic acid (2:1).

Mobile phase no. 8: 1-propanol:25% ammonia (11:9).

2.3.6 Development and drying of TLC plates

To saturate the chamber, 22.5 ml mobile phase was placed in each flat-bottomed TLC chamber for 10 x 10 cm plates 30 minutes before the development of the TLC plate. The chamber was sealed with parafilm and covered with a steel lid. The plates were developed over a path of 8 cm. The TLC plates were air-dried at room-temperature for minimum 1 hour before derivatisation.

2.3.7 Preparation of derivatisation reagents

The preparation of the derivatisation reagents is described in Table 7-14.

Table7: Derivatisation reagents for detection of carbohydrates.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
AA Naphthoresorcinol – sulfuric acid (Mod. Merck, 1980, pp. 59).	Solution a: 0.2 % naphthoresorcinol in 96% ethanol. Solution b: 20% sulfuric acid. Spray solution: Prepare freshly before use a mixture of equal parts of a and b.	Spray and heat for 7 min. at 105 °C.	Video documentation: White light. Video evaluation: Absorption.
AD$_\beta$ β-Naphthol – sulfuric acid (Mod. Merck, 1980, pp. 60).	10.5 ml 15% ethanolic solution (96%) of β-naphthol, 6.5 ml 96% sulfuric acid, 40.5 ml ethanol, and 4 ml water.	Spray and heat for 5 min. at 100 °C.	Video documentation: White light. Video evaluation: Absorption.
AI Thymol – sulfuric acid (Merck, 1980, pp. 93).	0.5 g thymol in 95 ml 96% ethanol and 5 ml 97% sulfuric acid.	Spray and heat for 17 min. at 120 °C.	Video documentation: White light. Video evaluation: Absorption.

Mod.: Modified according to.

Table 8: Derivatisation reagents for detection of P-containing compounds.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
BH Ammonium molybdate - tin(II)chloride (Mod. Merck, 1980, pp. 5).	Solution a: 1% ammonium molybdate in water. Solution b: 1% tin(II)chloride in 10% hydrochloric acid.	Spray with a and wait for 5 min. before spraying with b.	Video documentation: White light. Video evaluation: Fluorescence.
BI Cobalt(II)chloride (Mod. Merck, 1980, pp. 21).	1% cobalt(II)chloride in 96% ethanol.	Spray.	Video documentation: White light. Video evaluation: Fluorescence.

Mod.: Modified according to.

Table 9: Derivatisation reagents for detection of N-containing compounds.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
AL Fluorescein – ammonia (Merck, 1980, pp. 41).	0.005% fluorescein in 0.5 N ammonia solution.	Spray.	Video documentation: UV-254 and UV-366 light. Video evaluation: Absorption with UV-254 light and fluorescence with UV-366 light.
C Ninhydrin (Mod. Merck, 1980, pp. 61).	Dip solution: 0.5% ninhydrin and 3% glacial acetic acid in 96% ethanol. Stab. solution: 1 ml saturated aqueous copper(II)nitrate solution in 0.2 ml 10% nitric acid and 100 ml abs. ethanol.	Dip and heat for 2 min. at 110 °C. Dip into stab. solution.	Video documentation: White light. Video evaluation: Absorption.
E Bismuth(III)nitrate – potassium iodide (Mod. Merck, 1980, pp. 39; Wagner *et al*., 1984, pp. 301).	Solution a: 0.85 g bismuth (III)nitrate in 10 ml glacial acetic acid and 40 ml water. Solution b: 8 g potassium iodide in 20 ml water. Stock solution: Mix equal parts of a and b. Spray solution: Mix freshly 1 ml stock solution with 2 ml glacial acetic acid and 10 ml water before use. Stab. solution: 5% sodium nitrite in water.	Spray with spray solution, wait for 5 min. and spray with stab. solution.	Video documentation: White light, immediately after derivatisation. Video evaluation: Absorption.

Mod.: Modified according to.

Table 10: Derivatisation reagents for detection of organic acids and lipids.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
AN Bromocresol green - bromophenol blue – potassium permanganate (Mod. Merck, 1980, pp. 12).	Solution a: 0.075% bromocresol green and 0.025% bromophenol blue in 96% ethanol. Solution b: 0.25% potassium permanganate and 0.5 % sodium carbonate in water. Spray solution: Prepare immediately before use a mixture of 9 parts of a and 1 part of b. The solution is stable for 5-10 min. only.	Spray.	Video documentation: White light. Video evaluation: Absorption.
AV 2',7'-Dichlorofluorescein (Mod. Merck, 1980, pp. 26).	0.2 % 2',7'-dichlorofluorescein in 96% ethanol.	Spray.	Video documentation: UV-366 light. Video evaluation : Fluorescence.
AX Fluorescein (Mod. Merck, 1980, pp. 41).	0.01% fluorescein in 96% ethanol.	Spray.	Video documentation: UV-366 light. Video evaluation: Fluorescence.
AY Rhodamine 6 G (Mod. Merck, 1980, pp. 77).	0.001% rhodamine 6 G in 96% ethanol.	Spray.	Video documentation: UV-366 light. Video evaluation: Fluorescence.
G Rhodamine B (Mod. Merck, 1980, pp. 77).	0.1% rhodamine B in 96% ethanol.	Spray.	Video documentation: UV-366 light. Video evaluation: Fluorescence.
N Bromocresol green (Merck, 1980, pp. 12).	0.05% bromocresol green in 96% ethanol.	Spray.	Video documentation: White light. Video evaluation: Absorption.

Mod.: Modified according to.

Table 11: Derivatisation reagents for detection of alcohols and phenolic compounds.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
V Iron(III)chloride (Merck, 1980, pp. 50).	5% iron(III)chloride in 0.5 mol/L hydrochloric acid.	Spray.	Video documentation: White light. Video evaluation: Absorption.

Mod.: Modified according to.

Table 12: Derivatisation reagents for detection of S-containing compounds.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
AÅ Methylene blue (Mod. Merck, 1980, pp. 56).	Solution a: 0.025% methylene blue in 0.025 mol/L sulfuric acid. Spray solution: Mix equal parts of a and 96% ethanol.	Spray.	Video documentation: UV-366 light. Video evaluation: Fluorescence.
BG Palladium(II)chloride (Merck, 1980, pp. 60).	0.5% palladium(II)chloride in water containing a few drops 25% hydrochloric acid.	Spray.	Video documentation: UV-366 light. Video evaluation: Fluorescence.

Mod.: Modified according to.

Table 13: Derivatisation reagents for detection of terpenoids.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
BB Phosphoric acid (Mod. Merck, 1980, pp. 68).	Solution a: 85% phosphoric acid/water (1:1). Spray solution: 15% solution a in 96% ethanol.	Spray and heat for 20 min at 120 °C.	Video documentation: White light. Video evaluation: Absorption.
YB Sulfuric acid (Mod. Merck, 1980, pp. 91).	5% sulfuric acid in 96% ethanol.	Spray and allow the chromatogram to air-dry for 15 min. Heat for 5 min. at 110 °C.	Video documentation: White light. Video evaluation: Absorption.
Z Zinc chloride (Mod. Merck, 1980, pp. 99).	30% zinc chloride in 96% absolute ethanol. Place the solution in an ultrasonic bath for 30 min.	Spray and heat for 1 hour at 105 °C.	Video documentation: White light. Video evaluation: Absorption.

Mod.: Modified according to.

Table 14: Derivatisation reagents for detection of several groups of compounds.

Derivatisation reagent	Preparation of derivatisation reagent	Derivatisation	Video documentation/Video evaluation
A Vanillin – sulfuric acid (Mod. Merck, 1980, pp. 98).	Solution a: 50% sulfuric acid in 96% ethanol. Solution b: 2% vanillin in 96% ethanol. Spray solution: Prepare freshly before use a mixture of 1 part of a to 10 parts of b.	Dip and heat for 3 min. at 120 °C.	Video documentation: White light. Video evaluation: Absorption.
B Diphenylboric acid 2-amino ethylester (Mod. Merck, 1980, pp. 36).	1% diphenylboric acid 2-amino ethylester in 5% polyethyleneglycol 4000 in 96% ethanol.	Spray.	Video documentation: UV-366 light. Video evaluation: Fluorescence.
BC Iodine – potassium iodide (Merck, 1980, pp. 49).	0.2 % iodine and 0.4% potassium iodide in water.	Spray.	Video documentation: White light, immediately after derivatisation. Video evaluation: Absorption.
F Molybdatophosphoric acid (Mod. Merck, 1980, pp. 57).	10% molybdatophosphoric acid in 96% ethanol.	Dip and heat for 3 min. at 120 °C. Treat with ammonia vapour for 3 min.	Video documentation: White light. Video evaluation: Absorption.
I Anisaldehyde – sulfuric acid (Mod. Merck, 1980, pp. 6).	0.5 ml anisaldehyde in 10 ml glacial acetic acid, 85 ml abs. ethanol, and 5 ml 97% sulfuric acid.	Dip and heat for 5 min. at 100 °C.	Video documentation: White light. Video evaluation: Absorption.
R Silver nitrate – ammonia (Mod. Merck, 1980, pp. 78).	2.08% silver nitrate in 96% ethanol.	Treat with ammonia vapour for 5 min. before dipping. Dip and heat for 7 min. at 105 °C.	Video documentation: White light. Video evaluation: Absorption.

Mod.: Modified according to.

The TLC plate was treated with a derivatisation reagent after development. The derivatisation reagent was applied either by spraying or by dipping the TLC plate.

2.3.8 Derivatisation by spraying

The derivatisation reagents were transferred to either plastic bottles with screw caps with dosing holes or glass sprayers attached to a compressed air line and sprayed onto the TLC plate.

2.3.9 Derivatisation by dipping

The derivatisation reagents were poured into glass dipping chambers for 20 x 20 cm TLC plates and the TLC plates were dipped into the reagent for 2 sec.

2.3.10 Post-treatment of derivatized TLC plates

Several of the derivatisation reagents need heating to obtain a colour reaction. Therefore, the TLC plates were placed on a TLC plate heater. The heating times and temperatures are presented in Table 7-14.

2.3.11 Video documentation

The TLC plates were air-dried for 30 minutes before they were photographed and stored digitally. TLC plates treated with derivatisation reagent E (Bismuth(III)nitrate - potassium iodide) and BC (Iodine - potassium iodide) respectively, had to be photographed immediately after derivatisation because single spots or all the zones underwent very rapid colour change. On the Reprostar 3, the desired illumination, UV-254, UV-366 or white light was chosen. Regarding illumination, see Table 7-14. When using UV-366 for fluorescence, the camera UV blocking filter was inserted to produce true colours of the image. The TLC plate was displayed on the monitor as a “live” image. All the adjustments were made until the optimum was reached. The image was then photographed and stored.

2.3.12 Video evaluation

The digital images of the chromatograms were evaluated with the program CAMAG VideoScan. The desired image file was opened and a number of plate properties were selected. The type of image, fluorescence or absorption, was chosen depending on the colour of the TLC plate and the colour of the spots. Image types are presented in Table 7-14. The number of tracks to be displayed was chosen. The track pattern and the R_f range was defined and locked and the image of the TLC plate printed out from a colour printer.

The selected individual track was transformed into its corresponding analog curve with belonging peak properties (R_f value, peak height and/or peak area) and computed.

2.3.13 Evaluation of the results

The captured image was subjected to a visual inspection on the computer screen. Differences in concentration of a compounds found in the different cultivation methods were evaluated to be 25%, 50%, 75% or 100%, and this was noted as +, ++, +++ and ++++, where each + signify a difference of 25%.

2.3.14 Presentation of the results

The results of the screening are presented individually for each crop. The differences found, are specified by the TLC system in which the difference is detected and the R_f value (and colour) of a compound in the system.

2.3.15 Illustration of the screening process

The different steps in the screening process are presented in Figure 4 below.

Figure 4: The screening process.


Plant material, apples


Preparation of juice, apples

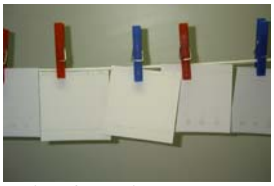

Centrifuging of juice, apples


Preparation of TLC plates

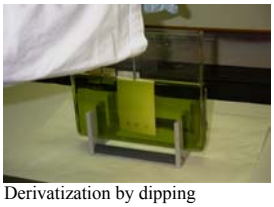

Preparation of mobile phases

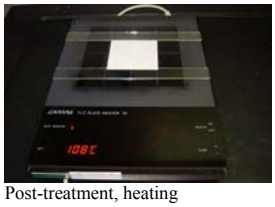

Application on TLC plates


Development of TLC plates


Drying of TLC plates

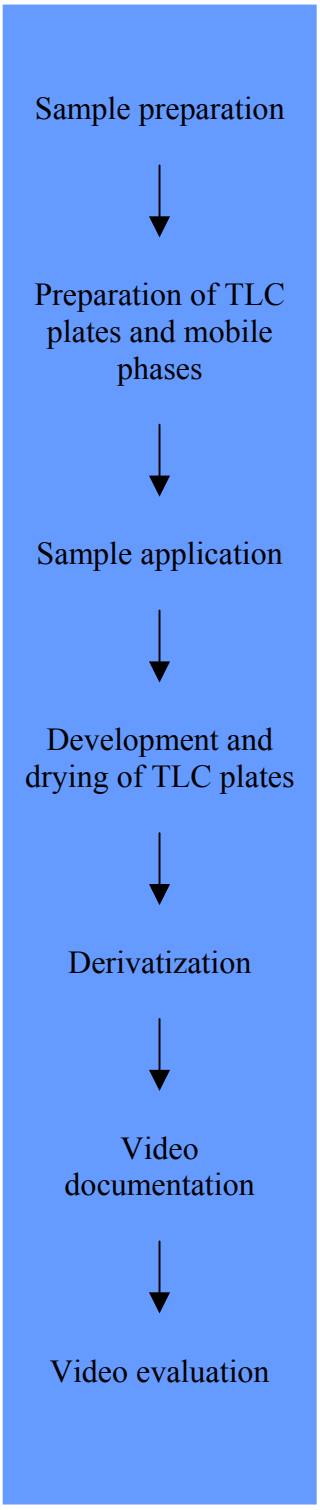

Derivatization by spraying


Derivatization by dipping


Post-treatment, heating


Video documentation


Video evaluation



3 Results and discussion

The presentation of this is divided in two parts. First the screening programme will be presented. Secondly, the results of the screening of the individual crops will be presentedand discussed.

3.1.1 Screening programme

The screening programme, developed on the basis of potatoes (*S. tuberosum* L.), is presented in Table 15.

Table 15: Screening programme.

Alcohols and phenolic compounds	Carbohydrates	N-containing compounds	Organic acids and lipids	P-containing compounds	S-containing compounds	Terpenoids	Several of the mentioned groups
V (52,8)	AA (47,1) AA (47,8) AD_{β} (47,8) AI (47,8)	C (47,8) C (52,2) C (52,8) E (47,1) E (47,8)	AN (47,1) AN (47,8) AN (52,1) AX (52,1) AX (52,2) G (47,1) G (47,8) G (52,8) N (52,1)	BH (47,1) BH (47,8) BI (47,8)	AÅ (47,1) AÅ (47,8) BG (52,1) BG (52,8)	BB (47,1) BB (47,8) YB (47,8)	A (47;8) B (47,1) B (47,8) F (47,1) F (47,8) I (47,1) I (47,8)

Capital letter: Indicates the derivatisation reagent, see table 16; **Numbers in brackets**: Indicate the stationary- and mobile phase; **1**:1-butanol:acetic acid:water (4:1:5), upper phase; **2**:1-butanol: 50% formic acid (2:1); **8**:1-propanol: 25%ammonia (11:9); **47**: Silica gel 60, HPTLC aluminium sheets; **48**: Silica gel 60 F_{254}, HPTLC aluminium sheets; **52**: Cellulose, TLC aluminium sheets.

3.1.1.1 Modification of the derivatisation reagents

Many of the derivatisation reagents were modified in relation to the literature. These modifications were done primarily since the solvents were toxic and easily could be replaced by less toxic solvents. As an example, methanol, being poisonous (Arbejdspladsbrugsanvisning, 1998), was replaced by 96% ethanol in derivatisation reagent BB (phosphoric acid).

3.1.1.2 Classification and specificity of the derivatisation reagents

A given plant contain a multitude of chemical compounds, which are products of either the primary or the secondary metabolism. The different phytochemical compounds were tried classified according to the biosynthetic origin of their principal constituents, see Table 16. This classification system was used by CBS Camag Bibliography Service Planar Chromatography (CAMAG, 2000) and Harborne & Baxter (1993).

Table 16: Classification of phytochemical compounds.

Alcohols and phenolic compounds	Carbohydrates	N-containing compounds	Organic acids and lipids	P-containing compounds	S-containing compounds	Terpenoids
- alcohols, phenols, phenolic acids and phenolic ketones - phenylpropanoids and derivatives - flavonoids and others	- mono-, oligo- and polysaccarides - sugar alcohols and others	- amino acids, amines, amino sugars - alkaloids - purines and pyrimidines, and other N-containing compounds	- mono-, di- and tricarboxylacids etc. - triglycerides, phospholipids, and glycolipids - unsaturated and saturated derivatives and others	- phospholipids and other P-containing compounds	- S-containing amino acids - sulfonamides - thiophosphate esters - sulfate esters of steroids and other S-containing compounds	- Monoterpenes, diterpenes, triterpenes, and sesquiterpenes - steroids - carotenoids and others

A literature search was carried out with the aim to identify the groups of phytochemical compounds detected by the individual derivatisation reagents, see Table 17. This was accomplished on the basis of the following literature:

- 1. CBS Camag Bibliography Service Planar Chromatography (CAMAG, 2000)
- 2. Dyeing reagents for Thin Layer and Paper Chromatography (Merck, 1980)
- 3. Thin Layer Chromatography (Stahl, 1969), and
- 4. Plant Drug Analysis (Wagner *et al*., 1984).

The literature search revealed that only few derivatisation reagents were specific, see Table 17. The majority of the derivatisation reagents detected many different groups of phytochemical compounds. Nevertheless, the derivatisation reagents were tried classified according to the group of phytochemical compounds predominantly detected, according to the literature, see Table 18. As an example, derivatisation reagent AX (fluorescein) detected organic acids and lipids and was therefore placed in this group, see Table 18. When a derivatisation reagent detects more than one phytochemical group and only one of the mentioned references described the detection of each phytochemical group, the derivatisation reagent was placed in the group "Several of the mentioned groups".

Derivatisation reagent F (molybdatophosphoric acid) detected organic acids and lipids according to CBS (CAMAG, 2000), Merck (Merck, 1980, pp. 57), and Stahl (Stahl, 1969, pp. 887), as well as terpenoids according to Merck (Merck, 1980, pp. 57), Stahl (Stahl, 1969, pp. 887), and Wagner *et al*. (Wagner *et al*., 1984, pp. 8). Therefore, the derivatisation reagent was placed in the group "Several of the mentioned groups", see Table 18.

Table 17: Derivatisation reagents used to detect different groups of phytochemical compounds.

Derivatisation reagent	Alcohols and phenolic compounds	Carbo-hydrates	N-containing compounds	Organic acids and lipids	P-containing compounds	S-containing compounds	Terpenoids
A: Vanillin – sulfuric acid	**X** $_{1,2,3,4}$			**X** $_{1}$			**X** $_{1,2,3,4}$
AA: Naphthoresorcinol – sulfuric acid		**X** $_{2,3}$					
AÅ: Methylene blue						**X** $_{2,3}$	
AD$_{\beta}$: β-Naphthol – sulfuric acid		**X** $_{2,3}$					
AI: Thymol – sulfuric acid	**X** $_{1}$	**X** $_{1,2,3}$					
AL: Fluorescein – ammonia			**X** $_{2,3}$				
AN: Bromocresol green – bromophenol blue – potassium permanganate				**X** $_{2,3}$			
AV: 2,7-Dichlorofluorescein		**X** $_{1}$		**X** $_{1,2,3}$			**X** $_{1}$
AX: Fluorescein				**X** $_{1,2,3}$			
AY: Rhodamine 6 G				**X** $_{1,2,3}$			**X** $_{1}$
B: Diphenylboric acid 2-aminoethylester	**X** $_{1,2,3}$						**X** $_{1,2,3,4}$
BB: Phosphoric acid	**X** $_{1}$		**X** $_{1}$				**X** $_{1,2,3}$
BC: Iodine – potassium iodide			**X** $_{3}$			**X** $_{1}$	
BH: Ammonium molybdate – tin(II)chloride					**X** $_{2}$		
BI: Cobalt(II)chloride	**X** $_{1}$				**X** $_{2,3}$		
BG: Palladium(II)chloride						**X** $_{2,3}$	
C: Ninhydrin		**X** $_{1}$	**X** $_{1,2,3,4}$	**X** $_{1}$			**X** $_{1}$
E: Bismuth(III)nitrate – potassium iodide			**X** $_{1,2,3,4}$	**X** $_{1}$			**X** $_{1}$
F: Molybdatophosphoric acid				**X** $_{1,2,3}$			**X** $_{2,3,4}$
G: Rhodamine B				**X** $_{1,2,3}$			
I: Anisaldehyde – sulfuric acid		**X** $_{1,2,3}$	**X** $_{1}$				**X** $_{1,2,3,4}$
N: Bromocresol green	**X** $_{1}$		**X** $_{2}$	**X** $_{1,2}$			
R: Silver nitrate – ammonia	**X** $_{1}$	**X** $_{1}$	**X**$_{3}$	**X** $_{1}$		**X** $_{3}$	
V: Iron(III)chloride	**X** $_{1,2,3,4}$						**X** $_{1}$
YB: Sulfuric acid		**X** $_{1}$	**X** $_{1}$			**X** $_{1}$	**X** $_{1,2,3,4}$
Z: Zinc chloride							**X** $_{2,3}$

1: CAMAG, 2000; **2**: Merck, 1980; **3**: Stahl, 1969; **4**: Wagner *et al*., 1984.

Alternatively, a literature search could have been performed, in order to determine the mode of action of the derivatisation reagents, but the mode of action of many derivatisation reagents not yet have been identified. Examples of this are: AI (thymol - sulfuric acid) (Jork *et al*., 1993, pp. 424) and YB (sulfuric acid) (Jork *et al*., 1990, pp. 412).

The overall modes of action of some derivatisation reagents, however, are known. For instance Iron(III)chloride, which reacts with phenolic compounds to give a coloured complex (Jork *et al*., 1993, pp. 272) and rhodamine B, which consists of an amino- and a carboxylic group that tend to form zwitter ions, which easily associate and accumulate in lipophilic zones of the chromatogram (Jork *et al*., 1990, pp. 401).

Derivatisation reagents included in the screening programme are classified in Table 19.

Table 18: Classification of the selected derivatisation reagents.

Alcohols and phenolic compounds	Carbohydrates	N-containing compounds	Organic acids and lipids	P-containing compounds	S-containing compounds	Terpenoids	Several of the mentioned groups
V	AA AD_{β} AI	AL C E	AN AV AX AY G N	BH BI	AÅ BG	BB YB Z	A B BC F I R

Capital letter: Indicates the derivatisation reagent (see Table 16).

3.1.2 Prospects for the screening programme

The screening programme will be used as a preliminary broad screening for phytochemical differences in plants exposed to stress compared to non-exposed plants, followed by selection and optimisation of the TLC systems, in which differences are found. A more simplified version of the screening programme may be used for instance, when investigating differences in one or several specific phytochemical groups.

In the nearest future, the screening programme will be applied in the screening of sea-grasses exposed to sulphur and TBT (tributyltin). Furthermore, different plants and root crops exposed to PAH (polyaromatic hydrocarbons) will be screened. Eventually, it will be determined if the screening programme can be used to detect biochemical differences in other living organisms exposed to stress, e.g. water fleas exposed to insecticides.

3.1.3 Presentation of the screening programme

The screening programme was presented as a poster at "Natur- og Miljø-forskningskonferencen" in Copenhagen 22-23 of August 2002.

3.2 Results and discussion of the screening of crops

3.2.1 Performed cultivation comparisons

The organically and the conventionally cultivated crops were compared as shown in Table 19-23. The phytochemical differences obtained when comparing conventionally- and organically cultivated kale (*B. oleracea* L.), carrots (*D. carota* L.), and peas (*P. sativum* L.) were tried explained in terms of soil conditions and fertiliser or the use of pesticides.

As far as potatoes (*S. tuberosum* L.) and apples (*M. domestica* Borkh.) are concerned, no real conventional cultivation was performed. The cultivation method resembling the conventional the most was performed on organic soil, resulting in a pseudo conventional cultivation method. Therefore, the results obtained for potatoes and apples may not reflect the phytochemical differences between actual conventional cultivation and organic cultivation.

Table 19: Comparison of different cultivation methods of potato (*S. tuberosum* L.)
Differences for a biomarker found between two parcels, is the result of the cultivation method stated in the cross square between the two parcels (see table 1).

Potato		Parcel 2
		Low N, Pesticide
Parcel 1	High N, Pesticide	**Fertiliser**
Parcel 3	Low N	**Pesticide**

Table 20: Comparison of different cultivation methods of peas (*P.sativum* L.)
Differences for a biomarker found between two parcels, is the result of the cultivation method stated in the cross square between the two parcels (see table 2).

Peas		Parcel 2
		Organically grown, Pesticide
Parcel 1	Conventional, Pesticide	**Soil treatment**
Parcel 3	Organically grown	**Pesticide**

Table 21: Comparison of different cultivation methods of kale (*B. oleracea* L.)
Differences for a biomarker found between two parcels, is the result of the cultivation method stated in the cross square between the two parcels (see table 3).

Kale		Parcel 2	Parcel 3	Parcel 4
		High N, Pesticide, conventional	Low N, Pesticide, Organically grown	Low N, Organically grown
Parcel 1	Low N, Pesticide, Conventional	**Fertiliser**	**Soil treatment**	Soil treatment/Pesticide
Parcel 2	High N, Pesticide, Conventional	-	Soil treatment/ Fertiliser	-
Parcel 3	Low N, Pesticide, Organically grown	Fertiliser/Soil treatment	-	**Pesticide**

Table 22: Comparison of different cultivation methods of carrots (*D. carota* L.)
Differences for a biomarker found between two parcels, is the result of the cultivation method stated in the cross square between the two parcels (see table 4).

Carrots		Parcel 2
		Low N, Organically grown, Pesticide
Parcel 1	High N, conventional, Pesticide	Soil treatment/ Fertiliser
Parcel 3	Low N, Organically grown	**Pesticide**

Table 23: Comparison of different cultivation methods of apples (*M. domestica* Borkh)
Differences for a biomarker found between two parcels, is the result of the cultivation method stated in the cross square between the two parcels (see table 5).

Apples		Parcel 2	Parcel 3	Parcel 4
		Low N	High N Pesticide	Low N Pesticide
Parcel 1	High N	**Fertiliser**	**Pesticide**	Fertiliser/ Pesticide
Parcel 2	Low N	-	Fertiliser/ Pesticide	**Pesticide**
Parcel 3	High N Pesticide	Fertiliser/ Pesticide	-	**Fertiliser**

3.2.2 Screening results

Differences in concentration of compounds found by screening were evaluated as stated in chapter 2.3.13 on page 27.

3.2.2.1 Potato (*S. tuberosum* L.)

Different cultivation methods were employed in the cultivation of potatoes (*S. tuberosum* L.). In all cultivations, organic soil was used. Consequently, none of the cultivation systems reflects the actual conventional cultivation method. The phytochemical differences found were related to the amount and type of fertiliser or to the use of pesticides (Table 19). See Table 1 for further details concerning the cultivation methods used. The results of the comparison are presented in Table 24.

Table 24: Phytochemical differences detected in *S. tuberosum* L. by use of TLC

Phytochemical compound detected	**TLC-system**	**R_f-value**	**Peel**			**Core**			**Related factor**
			Parcel 1	**Parcel 2**	**Parcel 3**	**Parcel 1**	**Parcel 2**	**Parcel 3**	
			High N, Pesticide	Low N, Pesticide	Low N	High N, Pesticide	Low N, Pesticide	Low N	
Terpenoid	**47-8-A**	**0.72**	+	+	++				**Pesticid**
	47-8-YB	**0.73**	+	+	++				
N-containing comp.	**47-8-C**	**0.70**				+	+	++	

TLC-systems: Stationary phase: No. 47, Silica gel 60, HPTLC aluminium sheets. **Mobile phase**: No. 8, 1-propanol:25% ammonia (11:9).
Derivatisation reagents: A: Vanillin-sulfuric acid (several groups), YB: Sulfuric acid (terpenoids), C: Ninhydrin (N-containing compounds)

Three biomarkers were found in potatoes, two in the peel and one in the core. There were indications that one biomarker (R_f value 0.72, brown spot) was detected in two TLC systems since the phytochemical differences detected with vanillin-sulfuric acid and sulfuric acid respectively, run in the same stationary- and mobile phase, had identical R_f values. It is difficult

to predict the nature of the biomarker, since the two derivatisation-reagents detect a wide range of compounds. According to Wagner *et al*., terpenoids have a strong blue, green, red and brown colour in the visible after treatment with vanillin-sulfuric acid (Wagner *et al*., 1984, pp. 8). Based on this, the biomarker found could be a terpenoid. The two biomarkers in potato were related to the use of pesticides.

3.2.2.2 Pea (*P. sativum* L.)

Three different cultivation methods were employed in the cultivation of peas (*P. sativum* L.) The phytochemical differences were related to the use of pesticides or cultivation method (table 20). See Table 2 for further details concerning the cultivation methods used. The results of the comparisons are presented in Table 25.

Table 25: Phytochemical differences detected in *P. sativum* L. by use of TLC.

Phytochemical compound detected	Date of screening	TLC-system	R_f-value	Parcel 1 Conventional, Pesticide	Parcel 2 Organically grown, Pesticide	Parcel 3 Organically grown	Related factor
N-containing comp.	**090402**	**47-8-C**	**0.28**	+	+++	+++	**Soil treatment**
	120402	**47-8-C**	**0.28**	+	++	+++	

TLC-system: Stationary phase: No. 47, Silica gel 60, HPTLC aluminium sheets. **Mobile phase**: No. 8, 1-propanol : 25% ammonia (11:9)
Derivatisation reagent: C: Ninhydrin (N-containing compounds)

One biomarker (R_f value 0.28, red spot) was detected with ninhydrin, detecting N-containing compounds. The biomarker could be arginine, since this amino acid has been identified as a red spot in the same TLC system with a R_f value of 0.24 (Nielsen, 2001). The biomarker found in peas was related to soil treatment of the parcels.

3.2.2.3 Kale (*B. oleracea* L.)

Different cultivation systems were employed in the cultivation of kale (*B. oleracea* L.). The phytochemical differences detected were related to the use of pesticides or to the soil treatment and the amount and type of fertiliser (table 21). See Table 3 for further details concerning the cultivation methods used. The results of the comparisons are presented in Table 26.

Table 26: Phytochemical differences detected in *B. oleracea* L. by use of TLC.

Phytochemical compound detected	Date of screening	TLC-system	R_f-value	Parcel 1 Low N, Pesticide, Conventional	Parcel 2 High N, Pesticide, Conventional	Parcel 3 Low N, Pesticide, Organically grown	Parcel 4 Low N, Organically grown	Related factor
Terpenoid	**160402**	**47-8-YB**	**0.50**	+++	++	++++	++	**Pesticid**
	220402	**47-8-YB**	**0.48**	++++	+++	++++	++	
Terpenoid	**160402**	**47-8-YB**	**0.59**	++	++	+++	+	
	220402	**47-8-YB**	**0.57**	+++	+++	+++	**n.d.**	
N-containing compound	**160402 & 220402**	**52-8-C**	**0.24**	+	+++	+	+	**Soil treatment/ fertiliser**

TLC-system: Stationary phase: No. 47, Silica gel 60, HPTLC aluminium sheets. **Mobile phase**: No. 8, 1-propanol : 25% ammonia (11:9)
Derivatisation reagents: YB: Sulfuric acid (terpenoids.), C: Ninhydrin (N-containing compounds)
n.d.: not detected

The biomarkers (R_f value approx. 0.49 and 0.58, brown spots) detected with sulfuric acid, may be terpenoid of origin, but this is uncertain, as the derivatisation reagent detects many groups of phytochemical compounds. The biomarkers were related to the use of pesticides. A biomarker (R_f value 0.24, violet spot) was detected with ninhydrin, which detect N-containing compounds. The biomarker could be related to the soil treatment and/or the amount and type of fertiliser.

3.2.2.4 Carrot (*D. carota* L.)

Three different cultivation systems were employed in the screening of carrots. The phytochemical differences detected were related to the use of pesticides or to the cultivation method (table 22). See Table 4 for further details concerning the cultivation methods used. The results of the comparisons are presented in Table 27.

Table 27: Phytochemical differences detected in D. carota L. by use of TLC.

Phytochemical compound detected	TLC-system	R_f-value	Parcel 1 High N, conventional, Pesticide	Parcel 2 Low N, Organically grown, Pesticide	Parcel 3 Low N, Organically grown	Related factor
N-containing comp.	**52-8-C**	**0.20**	+++	+	**n.d.**	**Pesticid**

TLC-system: Stationary phase: No. 52, Cellulose, TLC aluminium sheets **Mobile phase**: No. 8, 1-propanol:25% ammonia (11:9)
Derivatisation reagent: C: Ninhydrin (N-containing compounds)
n.d.: not detected

One biomarker (R_f value 0.20, purple spot) was detected with ninhydrin, detecting N-containing compounds. The biomarker found in carrots was related to the use of pesticides

3.2.2.5 Apple (*M. domestica* Borkh.)

Different cultivation methods were employed in the cultivation of apples (*M. domestica* Borkh.). The phytochemical differnces were related to the use of pesticides or fertiliser (table 23). See Table 5 for further details concerning the cultivation methods used. The results of the comparison are presented in Table 28.

Table 28: Phytochemical differences detected in M. domestica Borkh. by use of TLC.

Phytochemical compound detected	TLC-system	R_f-value	Parcel 4 Low N Pesticide	Parcel 3 High N Pesticide	Parcel 1 High N	Parcel 2 Low N	Commentary	Related factor
N-containing comp.	**52-8-C**	**0.37**	++	++	**n.d**	++	**purple**	**?**
Phenolic compound (flavonoid)	**47-1-B**	**0.68**	++	**n.d.**	++	++	**weak orange**	**N /Pesticid**
Organic acid/ lipid	**52-1-AX**	**0.50**	**n.d.**	**n.d.**	++	++	**-**	
Carbohydrate	**47-8-AA**	**0.71**	++	**n.d.**	++	++	**-**	
Terpenoid	**47-8-BB**	**0.71**	+	**n.d.**	++	++	**-**	
Organic acid/ lipid	**47-1-AN**	**0.05**	+	++	+	++	**weaker differences**	**?**
	52-1-AN	**0.05**	+	+	**n.d.**	+		
	52-1-AN	**0.15**	+	+	+	**n.d.**		
Phenolic compound	**47-1-B**	**0.61**	++	+	++	++		
	47-1-B	**0.65**	++	+	++	++		

TLC-systems: Stationary phases: No. 52, Cellulose, TLC aluminium sheets; No. 47, Silica gel 60, HPTLC aluminium sheets **Mobile phases**: No. 8, 1-propanol:25% ammonia (11:9), No. 1, 1-butanol:acetic acid:water (4:1:5), upper phase **Derivatisation reagents**: AA: Naphothoresorcinol-sulfuric acid (Carbohydreates), AN Bromoceresol green - bromophenol blue – potassium permanganate (organic acids/lipids), AX: Fluorescein (organic acids and lipids), B: Diphenylboric acid 2-aminoethylester (several groups), BB: Phosphoric acid (Terpenoids)
n.d.: not detected

Several biomarkers were found in apples. One biomarker (R_f value 0.68, orange spot) was detected in UV-366 nm with diphenylboric acid 2-aminoethylester. According to Sherma & Fried, diphenylboric acid 2-aminoethylester produces yellow and orange spots for flavonoids in UV-366 nm. (Sherma & Fried, 1996, pp. 719). The compound may therefore be a flavonoid. There were indications that one biomarker (R_f value 0.71) was detected in two TLC systems since the phytochemical differences had identical R_f values in two TLC-systems (derivatisation-reagents AA and BB) run in the same stationary- and mobile phase. The chemical nature of the biomarker is difficult to predict, since the two derivatisation-reagents detect different compounds. In addition to the above mentioned biomarkers, an organic acid or lipid (R_f value 0.50) was detected in UV-366 nm with fluorescein. All these biomarkers could be related to the use of pesticides or fertilisers. Four other biomarker were found in apples (table 28), but for these it was not possible to find a relation to the individual tretments.

3.2.4 Discussion of the experimental conditions

Extracts of potatoes (*S. tuberosum* L.), apples (*M. domestica* Borkh.), and carrots (*D. carota* L.) were prepared from a given number of units, for example extract of potatoes was prepared from 5 potatoes. To weigh out a certain amount of potatoes, apples, and carrots as it was done for kale (*B. oleracea* L.) and peas (*P. sativum* L.) would have been an advantage, to eliminate the

influence of varying water content in the crops. The dry weight of the crops could have been determined, or the crops could have been freeze-dried followed by extraction with ethanol. Fresh and fresh frozen material was simply pressed and the juice used for phytochemical analysis. Extracts were prepared this way since previous experiments showed a larger number of phytochemical compounds in fresh and fresh frozen pressed plant material compared with extracts prepared by extraction of freeze dried plant material with 75% ethanol. However, higher separation efficiency and better resolution of the phytochemical compounds was obtained using 75% ethanolic extracts, making it easier to detect possible phytochemical differences (Ravn, personal communication). In relation to developing a kit, extracts prepared by pressing fresh or fresh frozen material should be used instead of extracts made by extraction.
Preliminary investigations, with ethanol as extraction medium in the range from 5 to 96% ethanol proved that 75% ethanol, was most suitable for extraction of plant material, in that the greatest amount of compounds were extracted (Kristensen, personal communication). Therefore, the sun-dried peas were extracted with 75% ethanol.

It was envisaged to use the advanced TLC equipment available in the screening procedure of the crops. However, the greater part of the extracts was to viscous to be applied using the fully automatic sample applicator. Therefore, the application was manually performed.

3.2.5 Discussion of screening results

Many phytochemical differences were found between the organically and conventionally cultivated crops. However, only few of these were seen in both replicates. As an example, more than 50 phytochemical differences increasing or decreasing more than 25% were detected comparing organically and conventionally cultivated kale (*B. oleracea* L.). Only three of these differences were seen in both replicates. For carrots (*D. carota* L.), potatoes (*S. tuberosum* L.), and apples (*M. domestica* Borkh.), the phytochemical differences, only observed in one analysis, could be explained by variation in water content in the crops. Another explanation could be a degree of biological variation, e.g. variation between the different potato tubers from the same plant and differences between potato tubers from different plants. Dissimilar exposure to natural- and anthropogenic stress factors could also explain phytochemical differences found in only one of the replicates. The following examples of this aspect is found in the literature: Differences in the amount of anthocyanins in fruits of apples (*M. domestica* Borkh.) grown in sunlight or shade

respectively (Merzlyak & Chivkunova, 2000). Changes in the carbohydrate content of kale (*B. oleracea* L.) in response to turnip root fly larval damage (Hopkins *et al*., 1995). The effect of drought and UV radiation on the phenol level in pea (*P. sativum* L.) (Alexieva *et al*., 2001), and changes in the levels of terpenes and carbohydrates in different sorts of carrots (*D. carota* L.) in response to mechanical stress (Seljåsen, 2001).

Different stress factors may cause a variety of responses. These responses can be additive, synergistic or antagonistic. This was demonstrated in the study by Alexieva *et al*. (2001) carried out on peas (*P. sativum* L.) stressed with UV-B radiation and drought. It was found that the two stress factors acted synergistically on the amount of phenols.
In this project, the phytochemical differences between pseudo conventionally and organically cultivated crops were related to use of pesticide or fertiliser, but it is not possible to predict if conventional soil would take part of an inter-relationship with fertilisers and pesticides.

Two phytochemical differences related to the use of pesticides were detected in kale (*B. oleracea* L.). Since kale was only treated with insecticides and fungicides, this indicates that these have an effect on plants even though plants are not their targets.

A multitude of stress factors with different modes of action can cause the same or at least similar overall responses to stress (Lichtenthaler, 1995). This was illustrated by an example from the literature where the glycoalkaloid concentration in *S. tuberosum* increased in response to drought stress, but various other stress factors e.g. weather and inadequate storage conditions could have produced similar effects. Therefore, the response to drought stress seen in potatoes was not unique to the specific stress factor (Bejarano *et al*., 2000). It cannot be predicted if the biomarkers observed for the different screened crops are unique to the specific stress factors. This is a matter of inferior importance, as long as the biomarker pattern is unique to the specific stress factor.

The studies described in the introduction only investigated one or two groups of phytochemical compounds in response to a given stress factor. As an example, mechanical stress on the terpene and carbohydrate level in different varieties of carrots (*D. carota* L.) was investigated by Seljåsen *et al*. (1995). The performed screening differs from the effect studies found in the

literature in that a broad range of groups of phytochemical compound are investigated as opposed to only one or two groups in the effect studies found in the literature.
In the literature, only a few systematic tendencies were found when comparing conventionally- and organically cultivated plant food. In organically cultivated plant food, a lower nitrate level, resulting in a smaller amount of N-containing compounds and a higher vitamin C (ascorbic acid) level was seen compared with conventionally cultivated plant food. (Woese *et al*., 1997 and Williams, 2002).
In this project, five biomarkers belonging to the group of N-containing compounds were found; one in each of examined crops. These phytochemical differences in peas and potato showed an increase in concentration of more than 25% in organically cultivated crops compared to conventionally cultivated crops. The two biomarkers in carrots and kale showed a decrease in concentration of more than 25% in organically cultivated plant food compared to conventionally cultivated plant food, while the difference in apples was harder to relate to a particular factor. These results neither proved nor disproved the theory of a smaller amount of N-containing compounds in organically cultivated plant food.
Regarding a higher vitamin C (ascorbic acid) level in organically cultivated plant food compared to conventionally cultivated plant food, nothing was concluded on the basis of this screening. The only organic acid biomarkers were found in apples (*M. domestica* Borkh.). One of these related to either the use of pesticides or fertilisers, and increase by at least 50% in apples grown without the use of pesticides. It was not possible to predict whether some of these biomarkers are ascorbic acid or other acids. Presuming that the biomarkers actually are ascorbic acid, an increase of organic acids in the organically cultivated plant food was to be expected, in accordance with the literature.
Studies comparing organically and conventionally cultivated potatoes, carrots, and kale, found that the content of vitamin C (ascorbic acid) was not affected by the two different cultivation systems (Warman & Havard, 1997 and Warman & Harvard, 1998). Regarding kale and carrots, no organic acid biomarkers were found in this screening. This is in accordance with the above-mentioned study.

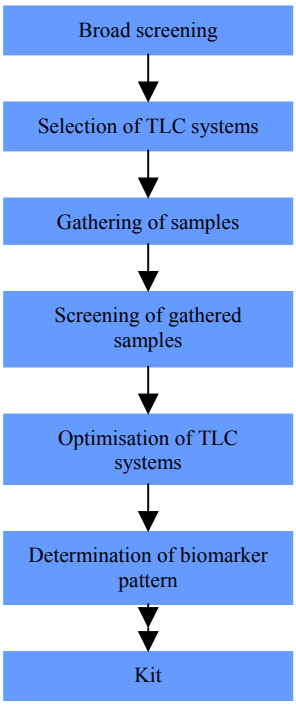
3.2.6 Discussion of conventional versus organic farming and health

The performed phytochemical screening of the crops revealed a number of phytochemical differences between the organically and conventionally cultivated crops. The chemical nature

and biological activity of the compounds differing will have to be determined, before it can be concluded to what extent these compounds are beneficial or harmful to health. The biological activity of the compounds differing can be determined in several ways. Isolation of the compounds followed by the use of different biological assays is one way to determine biological activity. Another way is to detect biologically active compounds directly on a TLC chromatogram. Spraying and coating techniques with an agar can be used, thereby making use of the fungicidal, antibacterial or antioxidative effects of the compounds to be determined (Hahn-Deinstrop, 2000, pp. 143). Haemolytic compounds can be detected directly on the chromatogram, by pouring a blood-gelatine solution onto the TLC plate (Hahn-Deinstrop, 2000, pp. 136).

3.2.7 Future development of a kit to determine whether crops are organically or conventionally cultivated using biomarkers

Figure 10: The different stages in the development of a kit.



The work towards developing a kit/kits to determine whether crops are organically or conventionally cultivated should continue.
The performed screening forms an essential part in the process of developing a kit. However, the results of the performed screening should be verified. As many samples as possible of organically as well as conventionally cultivated apples (*M. domestica* Borkh.), potatoes (*S. tuberosum* L.), peas (*P. sativum* L.), carrots (*D. carota* L.), and kale (*B. oleracea* L.) should be gathered and screened. Only the TLC systems in which differences were found in this project should be screened. If necessary, the screening should be followed by an optimisation of the TLC systems. It should be determined whether a common biomarker pattern valid for all species of the tested crops can be determined. Finally, efforts should be made to develop a simple kit.

The different steps in the development towards a kit are illustrated in Figure 10.

4 Conclusion

A broad screening programme, covering the most general phytochemical groups of compounds (e.g. terpenoids, lipids, phenolic compounds etc.), was developed on the basis of Thin Layer Chromatography (TLC). A total of 46 TLC systems comprising 26 derivatisation reagents, 3 stationary phases, and 4 mobile phases were included. The screening programme was applied in the screening of potatoes (*S. tuberosum* L.), peas (*P. sativum* L.*)*, kale (*B. oleracea* L.), carrots (*D. carota* L.), and apples (*M. domestica* Borkh.) grown in models of conventional or organic cultivation systems.

Distinctive phytochemical differences were found between the differently cultivated samples of these crops. In peas and carrots only one biomarker was found. In peas the biomarker was related to the soil conditions, while the biomarker in carrots was related to the use of pesticides. In potato, two biomarkers related to the use of pesticides were found. Three biomarkers were found in kale. Two of these could be related to the use of pesticide, while the last was related to either fertiliser or soil conditions. Several biomarkers were found apples, but a relation to the cultivation methods was not clear. Three of the biomarkers in apples could be related to either the use of pesticides or fertiliser, while no conclusions could be drawn from the other biomarkers found.

The results of this screening neither proved nor disproved the general tendencies found in the literature, showing a higher vitamin C (ascorbic acid) level and a lower level of N-containing compounds in organically cultivated crops compared with conventionally cultivated crops.

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Appendix