Inventarisation

LBI crystallisation work

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   Does the pipetting order effect the crystallisation picture?  
   Can different production systems (organic versus conventional) be discriminated?  
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1 THE TRIANGLE BIOCRYSTALLISATION APPROACH

1.1 Scientific background

Holistic methods for measuring the quality of food products have their background in the ontological assumption of a non-atomistic holistic ‘inner order’ in living organisms. This ordering structure organizes and structures the organism, is organism-specific and can be more or less harmonious, reflecting the state the organism is in under the conditions in which it lives and develops (Baars & Baars, 2007). This ‘inner order’ is believed to be connected to the growth and development (i.e. farming system) of the organism. Different holistic methods are regarded to reflect this ‘inner order’.

The Louis Bolk Institute participates within the international Triangle network (LBI, the Netherlands; BRAD, Denmark and Uni Kassel, Germany) in the standardisation and validation of the biocrystallisation method; a so-called (holistic) picture forming method. Parallel to this work the Steigbild or Capillary dynamolysis method, a second picture forming method, is standardised too.

Crystallisations are obtained by mixing a water soluble fraction (juice or extract) of a food product with copper chloride. This mixture is allowed to evaporate and finally crystallise under controlled conditions. Reference series show a clear correlation between certain aspects of the crystallisation pictures and the way the product was produced.

The standardisation of the method entails developing standardised crystallisation chambers, procedures and evaluation tools. The evaluation is based on computerised image analysis and Visual evaluation.

The Visual evaluation (section 4.1) is developed according to the ISO standards used for sensory analysis. In this way, 14 textural and structural morphological criteria have been developed and validated.

At the Louis Bolk Institute, the interpretation of the Visual evaluation is connected to the Inner Quality Concept (Bloksma et al. 2003). This concept is based on the universal life-processes Growth and Differentiation and the balance or Integration between these processes. A crystallisation is regarded to relate to good product quality when the characteristics of both life-processes are sufficiently present in the crystallisation and are found in a balanced manner (Integration). The computerised image analysis (section 4.2) increases the objectiveness of the method and allows the analysis of large numbers of crystallisations. The currently applied textural image analysis analyses the spatial variation in pixel grey values which however is not consistent with visual evaluation criteria and the morphological aspects found in the biocrystallisations. Therefore Structure Analysis software is developed which enables the characterisation of the crystallisation structure according to morphological features (Doesburg and Nierop in preparation).

1.2 General sample preparation approach

After receiving the products at the laboratory, sample preparation is performed. For the crystallisation of the products water soluble components are required. These are obtained
either directly (i.e. milk and juices); via an extraction procedure in distilled water (i.e. grains and cheese) or via a juice extraction procedure (vegetables and fruit).

The applied concentration ratio extract-CuCl$_2$ depends on the product under study. In a matrix, in which different extract-CuCl$_2$ concentration ratios are used, the optimum combination of concentration ratios is assessed visually. Depending on the research question, sample preparation is performed once or several times. Each sample preparation is pipetted in 3-5 fold replicate in the crystallisation chamber. Crystallisations are performed on one day, or repeated over several days, depending on the research question.
2 Calibration of the biocrystallisation chamber

2.1 Evaporation time estimation and standardisation

The time elapsed to the onset of the crystallisation (evaporation time) and the completion of it (crystallisation time) influence the morphological features characterising the crystallisation pictures. Therefore both times are determined for all crystallisations. The crystallisation chamber is calibrated at a median evaporation time of 13.5 ±1 hours (see section 25) with a freeze dried wheat meal chamber standard (90-90 mg in 6.0 ml). This standard is crystallised each experiment in 6-7 fold replication. Experiments and conditions are documented in LabDoc (Triangle laboratory documentation software for crystallisations).

2.2 Standard operating settings

The standard operating settings of the biocrystallisation chamber are:

- T measured directly below the plate = 30.0°C ± 0.2°C. For this a carton replicate of the glass plate (d=9cm) is used with a centrally positioned gap. Through this gap the T/rH sensor can be positioned directly below the ‘glass plate’.

- T outer room = 26.0°C ± 0.2°C.

- A median evaporation time of 13.5 ±1 hours of a freeze dried wheat meal chamber standard (90-90 mg in 6.0 ml). Adjustment of the 50% evaporation time is possible by altering the rH of the outer room.

2.3 Preliminary standard control-card; a multi-rule quality concept

This section is preliminary. At present all runs are regarded as ‘in-control’. The procedure below is based on the use of 2 standards, while currently only one is used. Evaluation of the crystallisation images originating from the standards is by means of textural image analysis. Therefore the variance coefficient cannot be determined (no ‘zero-point’ can defined), and no correlation is found with the main determinant of variance i.e. the crystallisation starting time. Presumably the structural analysis approach can overcome some of these problems. Besides this we don’t know what the method is measuring (self-organisation?), so there are no calibration possibilities (e.g. for spiking). Finally the present standard includes a laboratory (extraction and filtration) procedure and therefore reflects not only the crystallisation process but also the laboratory procedure.

The criteria to determine whether a run is in-or out-of control with respect to the standard, are based on the multi-rule quality control concept (Westgard 1981). An essential aspect to this is a continues quality control of the data arising from the biocrystallisation method i.e. the functioning of the biocrystallisation chamber.

- The biocrystallisation chamber controls consist of freeze dried wheat (flour)-CuCl$_2$. The working concentrations comprising 90-90mg in 6ml (control 1) and 70-90mg in 6ml
(control 2; or rather both consisting of a different wheat variety). Both controls require a separately performed wheat extraction. The controls are included in each experiment.

- Control data is obtained via image analysis of the scanned biocrystallisations. The ROI and variable combination for the Texture analysis of the chamber standards is set at ROI90 (analysing the majority of the crystallisation surface) and variable ‘sum variance’.
- Assumption: The error is distributed in a Gaussian manner.
- Due to a restricted chamber capacity, the chamber control μ and SD are determined on the basis of n=12 individual chambers (normally n>=20). For this, during each run a restricted number of plates is pipetted (3-4 per control).
- As up to date the influence the atmospheric pressure has on the crystallisation process hasn’t been evaluated, these n=12 runs must comprise a day with a high and a low atmospheric pressure.
- The criteria to determine whether a run is in-or out-of control are based on the multi-rule quality control according to Westgard (1981), in which the control data is displayed on a Levey-Jennings control chart (Levey and Jennings, 1950). The objective of these rules is to obtain a low level of false-rejections combined with an improved capability for detecting analytical errors, including some indication of the type of analytical error occurring when a run is detected, to aid in problems solving.

2.3.1 Control rules

1_{3SD}
Represents the control rule where a run is rejected when one control observation exceeds its control limit set as μ ±3SD. This is regarded as a random error.

2_{2SD}
Represents the control rule where a run is rejected when both controls exceed either their μ +2SD, or μ -2SD. This rule is also applied to two consecutive runs on the same control. This type of error is indicative for systematic errors; recalibration of the chamber may be necessary.

R_{4SD}
Represents the control rule where a run is rejected when one control measurement in a run exceeds its μ +2SD control limit, and the other control exceeds its μ -2SD limit. This “range” rule is sensitive to changes in the width of the distribution, Therefore it is a good indicate of increases in random error or changes in the precision of the method.

4_{1SD}
Represents the control rule where the run is rejected when four consecutive control observations exceed the same limit, which is either four times μ +1SD or μ -1SD. These consecutive observations can occur within one control (=four consecutive runs) or across controls (= two consecutive runs). This type of error is indicative for systematic errors; recalibration of the chamber may be necessary.

10_{μ}
Represents the control rule where the run is rejected when ten consecutive control observations fall on the same side of the mean. These consecutive observations can occur within one control (= ten consecutive runs) or across controls (= five consecutive runs). This type of error is indicative for systematic errors; recalibration of the chamber may be necessary.


3 DEVELOPING NEW PROCEDURES; STANDARDIZATION AND CHARACTERIZATION

Evaluation of large datasets required for the validation of procedures is currently restricted to textural image analysis. Textural image analysis does not yield a meaningful 'zero-value' for the parameters. Therefore the variance coefficient cannot be determined, which is one of the requirements for procedure validation. Besides this the standard used to determine whether a run is 'in control' is not optimal and interpretational yet. Therefore procedures can only be standardized and characterized at present. With a validated procedure the standard error rate is known, which allows a major decrease in sample preparation repetitions (day repetitions) to obtain significant results. At present the crystallisation process (most likely the evaporation time variation) is the biggest source of variation.

3.1 Method development and standardisation

When we start with a new product class, an existing protocol is used from a product class which is similar to the new one (e.g. triticale follows the wheat procedure). A literature search is performed (CuCl\textsubscript{2} related articles but also other methods yielding watery extracts).

1. Certain steps in the procedure are adapted for the new product.
2. A matrix is performed consisting of different mixing ratios in order to reach the area of the optimal picture (here only determined visually). This probably has to be repeated until the optimum is reached (evaluated visually). The final matrix should be performed with two samples which form a polarity in quality. If no satisfying discrimination is obtained at the optimal mixing ratio, this step is repeated.
3. Possibly some steps in the sample preparation have to be adapted if no satisfying pictures can be produced with the procedure (e.g. only multicentered pictures, only substance spirals etc.).

3.2 Method characterization

1. Before the repeatability of the method is determined, the chamber must be calibrated (e. g. 50% evaporation time for freeze dried wheat grist should be 13.5h +/- 1h). Adapting the relative humidity takes roughly 2-3 weeks to have an effect (to be adapted after the Checking Influence project 2009). So calibration should be performed minimally 2-3 weeks in advance. Maximal time since last calibration is ....(CI-report).
2. The repeatability of the method is determined by carrying out 6 times sample preparation and 6 pictures per each sample preparation on 3 days (day-factor) for the selected mixing ratio. This will be analysed by texture analysis and gives the variation of the method. When a substance vector is applied (3 concentration ratios) 2 times sample preparation for each of the 3 mixing ratios, with 6 pictures per sample preparation is performed. This is repeated on another day (best would be on 3 days in total).
3. This variation is compared with the quality difference (texture variables) between the different samples. This gives the information of how often repetitions have to be made.
The variation is calculated as the standard deviation of the certain step from those texture variables, which we apply for differentiation. We have the total standard deviation and the part of the sample preparation (when 3 times a day), the day (when > 3 day) and the chamber (when around 6 pictures per sample preparation, see JK habilitation page number 161).

4. The influence crucial steps in the procedure have on the result are analysed. This is done by repeating each step for one sample 4-6 times which gives the influence of the step on the variation (because the crystallization has the biggest influence it may not be necessary to do this here, it depends on the result from the repeatability testing).

5. Next to this the influence a change in the procedure has is tested (robustness test). This is performed on two or more samples in parallel and 2-3 repetitions at minimum (this is one of the characterizations methods described in ISO 17025).

3.3 Application

Different samples are analysed with repetitions in sample preparation, chamber and day according to the characterization results.

3.3.1 Results

After performing 3.1 and 3.2 we have a single-laboratory validation. The significance of the difference between samples can be tested. But this is just for the sample material tested (e.g. the wheat procedure cannot be transferred to barley, for barley most of the steps have to be done again).

Validation with the texture analysis means a differentiation ability of the method on a nominal scale (so just looking for differences, no ranking etc!).

3.3.2 Remarks

Repeatability tests and day-to-day variation should only be performed ones, as long as the conditions and the mixing ratio are not changed. If so, they have to be repeated.
4 EVALUATION OF THE CRYSTALLISATION IMAGES

4.1 Visual evaluation

Visual evaluation is based on the criteria for conventional profiling, which are developed according to the adapted ISO-Norm 11035 for sensory evaluation (Huber et al. 2009, submitted). Both quantitative and qualitative descriptive morphological criteria, as well as qualitative descriptive criteria of a higher order (gesture) are used. See figure 4.1.1 for some morphological criteria of crystallisations. Visual evaluation is either performed directly on the crystallisation plates, placed on a dark field illumination lightbox, or indirectly by use of photos of the crystallisation pictures. Visual evaluation procedures applied include conventional profiling (section 4.1.2), grouping (section 4.1.3) and ‘Two-group-testing’ (section 4.1.4). All 3 visual evaluation procedures can serve as a basis for the interpretation according to the Inner Quality Concept.

figure 4.1.1. A biocrystallisation with some morphological criteria depicted.

4.1.1 Photographing the crystallisations

Crystallisations are photographed with an Olympus E300 digital camera, fitted with a 14-45mm Zuiko Digital lens. Camera software version 1.0, Record mode HQ (JPEG file format 1/8 compression), Aperture priority, F10.0. Centre weighed average metering, Sharpness +1; saturation +1; contrast +2, White balance 4500K, ISO 200, Exposure compensation +0.3EV, Focusing mode single AF.

The crystallisations are photographed on a dark field illumination lightbox (dimensions 390x390x160mm lxwxh). Two 8 Watt/33 cool white TL-tubes are positioned 45mm from the sides of the lightbox (contact points at 55mm height). Two 390x60mm (lxh) pieces of multiplex (18mm thick) are positioned 90mm from the two sides of the lightbox (155mm apart from...
each other); providing the dark field illumination. All the wood is painted matt black to increase the illumination effect. The lid consists of a 4mm thick glass plate covered with matt black foil, with a centrally positioned 100mm diameter opening in the foil. Crystallisation plates are placed over this spacing, and covered with an 90mm diameter black mask.

4.1.2 Conventional profiling

Conventional profiling of crystallisations uses the 14 criteria (or a sub-set of these criteria) developed according to the adapted ISO-Norm 11035 for sensory evaluation (Huber et al. 2009, submitted). The criteria are ranked according to an ordinal scale of intensity, ranging from 1 to 9 on the basis of carrot crystallisation pictures, allowing a quantification of the observed profiles. Specific rankings may be needed for different product groups and/or treatments.

4.1.3 Grouping

Visual evaluation of crystallization pictures is generally performed by assessing first the impression of the typical character or ‘Gestalt’ unity, which the whole picture brings forward, followed by discriminating the different underlying morphological features. Perceiving this ‘Gestalt’ is enhanced when all crystallisation-replicates originating from one sample are evaluated simultaneously. The ‘Gestalt’ is commonly less manifest in single crystallisations due to extraneous factors like crystallisation starting time, positioning of the crystallisation centre, multi-centeredness, etc.

Grouping analyses whether the evaluator(s) can group different sample preparation repetitions (i.e. sub-samples) correctly on the basis of the ‘Gestalt’, as perceived in the crystallisation-replicates from one sample preparation. For this, the crystallisation-replicates belonging to each sample preparation are ordered according to the crystallization starting time, followed by a characterization and comparison with the crystallisation-replicates originating from the other sample preparations. This ultimately results in the grouping into sample-groups. When the evaluators are confident about the grouping, an overall characterisation is given for the different sample groups.

4.1.4 Two-group-testing

‘Two Group Testing’ is a visual classification method with which the statistical significance of the classification of crystallisations originating from 2 samples is tested. For this the crystallisations are treated as independent identically distributed random variables. That is, each random variable (i.e. crystallisation image) has the same probability distribution as the others and all are mutually independent.

Samples consisting of 10-20 crystallisations are drawn (adding up to a total of 100 crystallisations) and classified. The Chi squared test for hypothesis testing is performed to determine the probability of the found outcome.

The hypothesized distribution is the cross product of the row and column marginals divided by the sample size (see figure 4.1.4.1). ‘Two Group Testing’ is applied to objectify and statistically back the outcome of conventional profiling.
To determine the p-value of the Chi squared test, both distributions are required. In excel CHI.TOE(TS(actual distribution cells: hypothesized distribution cells).

4.2 Computerized Image analysis

The computerised image analysis increases the objectiveness of the method and allows the analysis of large numbers of crystallisations. The currently applied Texture analysis tool evaluates the pictures on their pixel grey-level distribution (a textural approach). As the crystallisation method yields pictures with a clear structure, attempts are being made to develop a structure analysis tool with criteria that resemble the Visual evaluation criteria. For both image analysis approaches scanned crystallisation images are used.

4.2.1 Scanning the crystallisations

The applied image analysis technique is described by Carstensen (1997), and concerning the specific application in a biocrystallisation context by Andersen et al. (1999). The pictures are scanned after storage of minimum 48 hours applying a UMAX PowerLook III slide scanner with 256 grey-levels.

4.2.2 Textural image analysis

For textural image analysis the RBG distribution is set to 33/33/33, and for pre-processing normalisation of the scans a Gaussian normalisation is applied. Analysis is performed on a circular Region Of Interest (ROI 1-100% of the crystallisation surface around the geometrical centre). For each crystallisation plate a Grey-Level-Co-occurrence-Matrix (GLCM) is calculated depicting the grey-level relationship between neighbouring pixels in the ROI. 15 variables characterising this GLCM are computed (Carstensen 1993). The 15 GLCM variables are divided into 3 groups. The variables within a group show a similar response between ROI and the p- and F-values. The 3 groups are represented by the following variables:

- Group 1: ‘Diagonal moment’
- Group 2: ‘Kappa’
5 resolution scales termed 1, 2, 4, 8 and 16 are available, yielding an increasing ‘smoothing’ of the images.

### 4.2.3 Structural image analysis

Structure Analysis software is developed which enables the characterisation of the crystallisation structure according to morphological features (Doesburg and Nierop in preparation). The present structure analysis software version 1 (SAV1) is able to quantify the crystal structure by computing the percentage of crystal coverage for 15 crystal-width ranges and the total coverage (e.g. d20 depicts a maximum crystal-width of 2 pixels; d24 depicts the crystal-width ranging from 2 to 2.4 pixels; etc. for d29, d35, d41, d48, d55, d64, d73, d83, d94, d107, d120, and d134, ending with d150 having a width ranging from 13.4 and above). The ranges are chosen in such a way that a clear visual transition in the biocrystallisation picture is obtained when the crystal structure is coloured according to these different widths. Similarly the crystal-free areas can be quantified, that is the areas in the crystallisation image where no copper chloride deposits are found. The analysis of the crystal coverage can be performed on the entire crystallisation plate or on certain Regions of Interest (ROIs) around the geometrical centre point of the crystallisation plate; ROI50 analyses 0-50% of the crystallisation plate, ROI70 analyses 50-70% and ROI90 70-90%. Parameter names consist of [characterised structure - ROI – Width range], e.g. CFROI70d94 denotes [crystal-free structure - ROI70 - width range d94] and d64 [crystal structure - entire plate - width range d64]. The structural image analysis characterises the crystal and crystal-free structure based on the coverage of 15 crystal widths and the total coverage, which in combination with 4 ROIs generates a basis of 128 SAV1 parameters. Data analysis is performed with Matlab software (version 7.7.0 R2008b, The Mathworks).

### 4.3 Statistical analysis

#### 4.3.1 ACIA; textural image analysis

ACIA uses variance analysis based on a linear mixed model for the statistical analysis of the textural analysis variables, and connects the scanned images to the sample preparation information (LabDoc). Output generates the p- and F-values for the 15 different variables relative to the ROI, represented as ‘p and F over ROI-plots’.

To assess the differentiation of multiple samples a wide range of ROIs (ROI 1-100%) and ROI shapes (circle; circle segment and circle in circle) can be used. Combined with the 15 textural analysis variables this results in an extensive ROI-variable combination set. The validity of the used ROI-variable combination is based on a non-significant Shapiro-Wilk (normality test) and Bartlett-test (testing homogeneity of variance) and a stable progression over ROI, as visualized in the ‘p and F over ROI-plots’. An additional validity check is the so called ‘grouping’ of (coded) subsamples; that is determine which samples the subsamples originate from. At present this is determined visually with the ‘p over ROI-plots’. If grouping is correct, this serves as an extra validation for the use of the ROI-variable combination.
4.3.2 Trend analysis and regression modelling

This approach can be performed on evaluation parameters from the above mentioned evaluation approaches (e.g. textural analysis, structural analysis; and visual evaluation conventional profiling), and on combinations of these.

Single-day analysis

Single-day analysis is used when experiments are performed on a single day only, or to determine the single-day sample prediction potential for experiments running over several days.

By means of stepwise regression, subsets of the available parameters are selected, predicting the samples for each experimental day. Potential regression models are generated using 1 to maximum 2 parameters. The following methods are applied: ordinary stepwise forward and stepwise selection with p-value to enter fine tuning (StepwiseForwardP and StepwiseP), Least Absolute Shrinkage and Selection Operator (Lasso), a rescaled version of the Lasso (ConeLasso) and Orthogonal Matching Pursuit (OMP). The accuracy is estimated with CVcorrect (cross-validated correct rate of classification, averaged over hundred 5 fold cross-validations). A final selection of reliable subsets of parameters is based on 999 random permutations of the images, assessing whether there is a significant 10% gap between the true $R^2$ and the distribution of all permuted $R^2$’s (B. Andersen et al. 2009; B. Andersen submitted). Random permutation tests resulting in a p-value below 0.05 are considered as a valid result.

Analysis of multiple days combined

In order to focus on relative differences between the days, it may be necessary to normalise the variables by subtracting the day-mean of the corresponding variable. The best differentiating parameters for each single day are determined by testing the mean differences between the sample groups with two-sample t tests with pooled variance (Kreyszig, 1970). In this way 4 parameters with the lowest p-values are selected for each day. Reflected Discriminant Analysis (RDA trend analysis) is performed to investigate the main trend in the data. RDA can be conceived as a PCA (principal component analysis) reflected by group information (Nierop, 1994). If this trend reflects the sample differentiation, a stepwise selection will be computed to investigate whether this can be determined with a lower number of variables too. Stepwise selection, accuracy estimation and validation of the model are computed as above.

Grouping of subsamples with Trend analysis

This can be performed on single days or on multiple days combined. When multiple days are analysed, it may be necessary to normalise the variables by subtracting the day-mean of the corresponding variable. This allows a focus on relative differences between the days. The best differentiating parameters for each single day are determined by testing the mean differences between the sample groups with two-sample t tests with pooled variance (Kreyszig, 1970). In this way 4 parameters with the lowest p-values are selected for each day. RDA trend analysis is performed to investigate the main trend in the data. In this way subsamples originating from the same sample can be grouped (if differentiation of the samples occurs).
5 GROUND COFFEE SAMPLE PREPARATION

5.1 Procedure

1. 50.0 gr ground coffee is transferred into a 500 ml wide-necked Erlenmeyer flask.
2. 450.0 ml of high purity water (25 °C) is added while simultaneously manually shaking the flask, thereby securing that all ground is in contact with the water. Subsequently the flask is covered with parafilm.
3. For extraction, the Erlenmeyer is placed on a shaker (Heidolph Unimax 2010) at 200 rpm for 30 min.
4. Subsequently the extract is left to stand for 15 min.
5. The total required volume of extract is pipetted approximately 2 cm below the surface level into a bekerglass.
6. Concentration ratios yielding pictures with a well defined ramification structure are 75-130 and 90-120 (subst-CuCl₂ in mg or µl); see figure 5.1.1.

![Figure 5.1.1. Concentration ratio 75-130 (2004.01.16.CM, 15-15, left) and 90-120 (2004.01.16.CM, 22-17, right). Simon Lévelt, café organico ‘Dolce’ mild Arabica terStart unknown.](image)

5.2 Research Question

*Does decaffeination effect the crystallization structure?*

A pilot was performed (2004.01.16.CM, see figure 5.3.1). A difference was observed by conventional profiling, that crystallisations from decaffeinated coffee ground are less filled, having fewer but longer sideneedles with a large ramification angle which frequently even is transverse. Furthermore decaffeination shows a tendency towards large hole-forms formation (Lemniscates). The overall impression is a ‘hardening’ of the crystallization structure after decaffeination. However, no analytical data substantiating this result is available, see figure...
5.3.1.

Is filtration of the coffee extract required

This was assessed in the same pilot as above (2004.01.16.CM). The extract was filtered over a Frisinette 0FF folded paper filter. Picture forming properties were reduced as a result, as determined by conventional profiling. Therefore filtration of the extract is not required.

Figure 5.3.1. two concentration ratios from experiment 2004.01.16.CM, demonstrating the decaffeination effect.
5.3 Recommendations for future research

- The protocol uses 25°C water for extraction. It might be worthwhile using boiling water in the extraction procedure combined with a shorter extraction time (5') followed by filtration over Whatmann #41 (20-25µm pore size).
- The recommended brewing temperature of coffee is 93°C. If cooler, some of the solubles that make up the flavour will not be extracted. If the water is too hot, some undesirable, bitter, elements will be extracted, adversely affecting the taste. If coffee is heated to boiling point only very briefly, the taste will be little affected; the longer it is kept at a high temperature the worse the taste becomes.
6 PREPARATION OF CRESS EXTRACTS TO ANALYSE THE EFFECT DIFFERENT SOLUTIONS HAVE ON GERMINATING SEEDS

6.1 Procedure

Seed selection:
1. For the germination, garden cress seeds (*Lepidium sativum*, supplier Bingenheimer Saatgut AG, article nr. G250) are used.
2. Selection of the cress seeds is based on the criteria size (very small/large seeds are discarded), colour (very light-brown/dark-brown seeds are discarded) and outer form (broken, damaged, irregularly shaped and insufficiently filled seeds are discarded).
3. In total 1100 seeds are selected and pooled. From this pool 60 cake cups are filled with 18 seeds each (in total 1080 seeds), leaving 20 seeds extra for replacing deviating seeds found in second instance.

Setting up the germination:
4. For each experimental day 6 samples can be analysed.
5. The germination takes place on chromatography paper, placed in plastic MiNiGRiP bags. The papers are placed with the fibre direction positioned downwards and the smooth side facing upwards (germination side) in the bags.
6. A code is written 3cm from the top concerning experimental day, sample and bag replicate (1-10).
7. A total of 10 bags are applied per sample.
8. A volume of 3.00ml is pipetted onto the paper. When the sample is absorbed evenly over the paper 18 seeds are placed 9-10 cm from the bottom of the paper.
9. A minimum access of air into the bag is arranged by manually expanding the upper closing area of the bag at both sides simultaneously.
10. The bags are placed in the germination pan hanging from stainless steel sticks. The 6 samples are positioned according to the numbers written on the outside of the pan (i.e. #1-3 on the first stick, and #4-6 on the other; so that #1 and #3 are positioned on the same side of the pan). The pan is covered with black carton/a plastic plate and placed at 19°C (+-1°C) in a heating cabinet on a perforated plate.
11. When the seeds have developed a mucous sphere after approx. 2.5 hours the seeds are positioned on a line 9cm from the bottom of the paper at 2-3mm distance. For this 16 seeds are required, leaving 2 extra seeds to replace seeds not forming a mucous sphere. Abundant seeds are discarded.
12. Again the bags are opened for a minimum access of air by expanding the upper closing area of the bags at both sides simultaneously. The pan is covered with black carton/a plastic plate and placed back into the heating cabinet on a perforated plate for approximately 96 h.

Preparation of seedlings for extraction:
13. After germination the bags are cut open in both sides, and on the top, approximately 3mm from the paper. The front side of the bag is opened, and the upper part of the paper is
bent backwards whereby the seedlings are accessible for manual selection of single seedlings.

14. Per sample a fresh pair of examination gloves is used to prevent sample contaminations.

15. Seedlings with a minimum root length of 6.5 cm are selected from each, generally resulting in 8-12 seedlings per bag. Seedlings which deviate in shape or colour, or which show signs of fungal growth, are discarded. The brown seed coats are not included.

16. The seedlings are placed in a Petri dish on a 3 decimal weight. The number of seedlings from each bag is noted on a standard form, together with the increasing weight of the selected seedlings, for statistical analysis.

17. A total of 3.00g seedlings is applied.

18. The seedlings are placed in a mortar containing 10.0 ml of sample. The mortar is covered with Parafilm until seedlings from all treatments have been prepared.

**Extraction:**

19. The seedlings are crushed by means of a pestle applying diagonal movements for 2 min whilst standing, whereby no intact leaf or root parts may be observed in the solution. Subsequently lemniscate movements are applied for 1 min whilst sitting whereby the leaf and root parts are diminished further.

20. The pestle and the mortar are each flushed with 8.50ml sample, thereby generating a 10% solution on weight basis (3.00g seedlings; 27.00ml sample; in total 30.00g solution).

21. The solution is transferred to a wide-necked 100 ml Erlenmeyer flask. Each flask is covered with Parafilm and left standing until all treatments are performed.

22. The flasks are extracted on a horizontal shaker (Heidolph Unimax 2010) at 125 rpm for 45 min.

**Preparation of the crystallisation solution:**

23. The extract is filtered for 3 min by means of a nylon filter with pore-size 150 µm placed in a glass funnel placed in a pre-weighed wide-necked 100ml Erlenmeyer flask. The weight of the filtrate is noted.

24. The Erlenmeyer flask is placed on a 3 decimal weight, and the weight of the filtrate is reduced by means of a Pasteur-pipette to 21.70g.

25. Into the remaining filtrate 21.00ml of a 5% CuCl₂ solution is pipetted representing a total of 7 crystallisation portions, each containing 3.10ml filtrate and 3.00ml CuCl₂ solution.

26. From each crystallisation solution 6 plates are pipetted with a **6.1ml** volume, this corresponds to 310mg substance/150mg of CuCl₂, see figure 6.1.1.
6.2 Research Question

*Can the effect of D30 potencies on germinating seeds be discriminated and characterised?*

When the experiments are repeated for several days a significant effect of the potencies can be found; see report ‘Development of biocrystallisation test system for examining effects of homeopathic metal potencies on germinating seeds; J-O. Andersen, P. Doesburg, M. Huber, S. Baumgartner; 2009 (in progress)’. Evaluation is based on conventional profiling, grouping, textural and structural image analysis.

6.3 Recommendations for future research

- The processing order of the 6 samples must be in such a way that they are evenly distributed over all 6 processing order positions for the different experimental days. This to circumvent any possible processing order effect.
- A processing order effect can probably be decreased by directly proceeding further after point 17 and 20 of the procedure.
White Cabbage Sample Preparation

7.1 Procedure

1. Samples consist of at least 4 cabbages, stored at 4-6 °C in the dark.
2. The outer leaves are taken off. One quarter of each cabbage is used for juice preparation. The quarter is cut into segments making sure not to add any cabbage stem material. Rotten parts are cut out.
3. Juice is produced with the ‘Greenstar’ juice machine fitted with the small sieve and the valve with the lightest resistance.
4. The two squeezers of the Greenstar mills are assembled in such a way that the dot of the first squeezer is positioned between the two dots on the second squeezer. The spring is pressed and de-pressed a couple of times and finally is screwed on until it reaches its resisting point. Before juice is collected from the first sample, the excess cabbage segments of this same sample are processed until the Greenstar is saturated and juice and pulp is produced.
5. Juice is collected from the 4 cabbage segments up to 1 minute after the last segment has been processed.
6. The juice is filtered over a 110 micrometer nylon filter into a 250 ml measuring cylinder.
7. The cylinder is covered with parafilm and the juice is left standing for 20 minutes at RT to allow the sedimentation of insolubles.
8. To prepare the Greenstar for the next sample, approximately 50 gr of cabbage (of the segments that are to be omitted) of the next sample is processed. This juice is discarded.
9. The settled juice is directly added to 100 ml wide-necked erlenmeyers already containing H₂O and CuCl₂.2H₂O, to prepare the necessary chamber solutions. For this the juice is pipetted approximately 2 cm under the surface level of the cylinder.
10. Concentration ratios yielding pictures with a well defined ramification structure are 410-220, 460-220 and 410-270 (subst-CuCl₂ in mg or µl), see figure 7.1.1.
7.2 Research Questions

Can different production systems (organic versus conventional) be discriminated?

As a pilot, a concentration matrix was performed (2004.03.19.wCM). Conventional profiling showed a big difference between the organic and conventional white cabbage crystallisations at concentration ratio 410-220. However, the tcrStart was not determined, only 1 replicate was produced per concentration ratio and no analytical data substantiating this result is available.

The main characteristics are that the conventional white cabbage crystallizations are less filled, having fewer but longer sideneedles with a large ramification angle which frequently even is transverse. Perradiation is stronger, so is the centre coordination whereas the curvature impresses as more rigid. The stem formation is more prominent. The crystallizations from the conventional sample impress as riper but also more rigid than the organic sample.

Of course the results from this pilot only reflect the difference between these two individual samples, of which the varieties and growth conditions are unknown. These results do not serve as a solid basis for the discrimination between organically and conventional produced white cabbages in general.
Can different varieties be discriminated?

This was assessed and confirmed on the basis of conventional profiling in experiment 2004.04.02.wCC; see report ‘Voedingskristallisaties van vier witte koolrassen’. 

Figure 7.3.1. Photos from experiment 2004.03.19.wCM, demonstrating the alleged effect of different production systems.

190304 13-13 410-220 org.JPG tcrStart unknown (410-220) Cryst. From an organic white cabbage sample

190304 14-14 410-220 conv.JPG tcrStart unknown (410-220) Cryst. From a conv. white cabbage sample
8 TOMATO SAMPLE PREPARATION

Note: pipetting order H2O-sample-CuCl2

8.1 Procedure

1. Samples consist of at least 30 tomatoes.
2. The samples are washed under tap water and dried with a towel.
3. One fourth of each tomato is used for juice preparation. The segments are cut longitudinally.
4. Juice is produced with the ‘Greenstar’ juice machine fitted without the sieve but with the valve with the light resistance spring.
5. Before assembly of the Green Star, all parts are wetted with mono-distillated water.
6. The two squeezers of the Greenstar mills are assembled in such a way that the dot of the first squeezer is positioned between the two dots on the second squeezer. The spring is pressed and de-pressed a couple of times and finally is screwed on until it reaches its resisting point.
7. The juice from the tomato segments is collected up to 1 minute after the last segment have been processed.
8. Before collecting juice from the next tomato sample the Green Star is dismantled and rinsed with mono-distillated water.
9. 200 gr of tomato pulp is poured into an Erlenmeyer after which the Erlenmeyer is filled up to 400 gr with 25°C bi-distillated water.
10. The flask is covered with parafilm and placed onto a horizontal shaker at 180 rpm for 30’.
11. Pour 80 ml extract onto a 110 micron nylon filter placed over a narrow-necked 250 ml Erlenmeyer flask.
12. The total filtration time is 10”.
13. The filtrate is added to 100 ml wide-necked Erlenmeyers containing only H2O, to prepare the necessary chamber solutions.
14. The necessary amount of 10% CuCl2 solution is added directly after which the solution is swirled by hand to homogenisation and covered with parafilm.

Concentration ratios yielding pictures with a well defined ramification structure are 620-140 and 755-170 (subst-CuCl\textsubscript{2} in mg or µl), see figure 8.1.1.
8.2 Research Questions

Can different ripening stages (unripe – ripe – overripe) be discriminated?

A clear ripening effect was found, which was characterized with conventional profiling; see report ‘Biokas, Tomaten kristallisaties September – Oktober 2004’ and experiment 2004.10.22.TC, see figures 8.3.1 and 8.3.2.

Is heating the greenhouse reflected in the tomato crystallisations?

A clear difference was found between tomatoes originating from a heated versus an unheated greenhouse, which was characterized with conventional profiling (see report ‘Biokas, Tomaten kristallisaties September – Oktober 2004’). The tomatoes originating from the unheated greenhouse were superior in their picture forming properties in combination with the inner quality aspects ‘growth’, ‘differentiation’ and ‘integration’. However, no analytical data substantiating this result is available. The samples originate from different greenhouses so other growth parameters, which were not standardised, may influence the outcome too. Therefore these results do not serve as a solid basis for the discrimination between tomatoes originating from heated versus unheated greenhouses in general.

Can different varieties be discriminated?

This was assessed and confirmed on the basis of conventional profiling; see report ‘Biokas, Tomaten kristallisaties September – Oktober 2004’. However, no analytical data substantiating this result is available.

Is the harvest moment reflected in the crystallisations?

This was assessed and confirmed on the basis of conventional profiling; see report ‘Biokas, Tomaten kristallisaties September – Oktober 2004’. Harvesting later in the growth season
shows an increase of degradation (overripe) related morphological aspects. However, no analytical data substantiating this result is available.

*Is a reduction in the watering reflected in the crystallisations?*

This was assessed on the basis of conventional profiling in report ‘Biokas, Tomaten kristallisaties Nov. 2005’. Reduced watering versus conventional watering was performed in the same greenhouse, on the same tomato variety. The product quality as determined by crystallisation increases when watering is reduced. However, this increase has a seasonal dependency.

![Figure 8.3.1](TC 22102004 20-02 620-140 unripe 12h01.JPG) ![Figure 8.3.1](TC 22102004 16-04 620-140 ripe 12h42.JPG) ![Figure 8.3.1](TC 22102004 06-06 620-140 overripe 14h43.JPG)

*Figure 8.3.1. Photos from experiment 2004.10.22.TC, demonstrating the ripening effect at concentration ratio 620-140.*
Figure 8.3.2. Photos from experiment 2004.10.22.TC, demonstrating the ripening effect at concentration ratio 755-170.
9 RED WINE SAMPLE PREPARATION

9.1 Procedure
1. The red wine is manually homogenized in the bottle.
2. 50 ml sample is poured into a bekerglass.
3. The required volume of wine is pipetted approximately 2 cm below the surface level.

Concentration ratios yielding pictures with a well defined ramification structure are 1600-250 and 1600-270 (subst-CuCl$_2$ in mg or µl), see figure 9.1.1.

![Figure 9.1.1. Concentration ratios 1600-250 (left, 2008.02.11.FH, 29-09, 12h40) and 1600-270 (right, 2008.02.11.FH, 08-07, 11h39). Sample Albert Heijn ‘Huiswijn’, 12%.

9.2 Research Questions

*Is the wine price reflected somehow in the crystallisation pattern?*

This was assessed for two wine samples. A difference was observed by conventional profiling; see experiment 2008.02.11.FH and figure 9.3.1. The crystallisation centres of the expensive wine crystallisations (Domaine Richeaume) are well formed and show a strong coordination for both concentration ratios tested. The expensive wine crystallisation overall structure also appears as more harmonic.

Of course the results from this pilot only reflect the difference between these two individual samples, of which the grape variety and production conditions aren’t determined. These results do not serve as a solid basis for the discrimination between alleged quality differences.
in general.

Figure 9.2.1. Photos from experiment 2008.02.11.FH, demonstrating the alleged price effect at concentration ratios 1600-250 and 1600-270.
10 PEA SAMPLE PREPARATION

10.1 Procedure

1. The samples are cleaned by means of a 2.0mm Retsch sieve, followed by manually removing of damaged and broken kernels and remaining foreign particles.
2. 100g of each sample is grinded with a centrifugal mill (Retsch ZM 100; 14.000 rpm) fitted with a 1.0 mm ring sieve.
3. 25.0g of the milled material is extracted in 475.0 ml deionised water (25°C) on a (Heidolph Unimax 2010) horizontal shaker at 200 rpm for 30 min.
4. Subsequently the extract is left to stand for 15 min.
5. Due to clogging of the normally used Whatmann filters, the pea extracts are filtered over a nylon sieve (pore size 20 micron).
6. Filtrates are added to CuCl$_2$ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 100-250 (subst-CuCl$_2$ in mg or µl); see figure 10.1.1.

![Figure 10.1.1. Concentration ratio 100-250 (2006.06.06.CI, 08-02, 14h02). Sample #52](image)

10.2 Research Questions

*Can different production systems (organic versus conventional) be discriminated?*

This was assessed in the project ‘Organic more healthy?’; experiments 2006.06.06.CI and 2006.06.09.CI. Two samples, consisting out of 3 subsamples each, were analysed on two
consecutive days. Grouping of the subsamples based on textural analysis grouping was correct, however the two sample groups could not be differentiated significantly.

10.3 Recommendations for future research

Determine whether the chosen concentration ratio is optimal for differentiation of samples. For this, two samples are required that form some kind of polarity.
11 SOY SAMPLE PREPARATION

11.1 Procedure

1. The samples are cleaned by means of a 2.0mm Retsch sieve, followed by manually removing of damaged and broken kernels and remaining foreign particles.
2. 100g of each sample is grinded with a centrifugal mill (Retsch ZM 100; 14.000 rpm) fitted with a 1.0 mm ring sieve.
3. 25.0g of the milled material is extracted in 475.0 ml deionised water (25°C) on a (Heidolph Unimax 2010) horizontal shaker at 200 rpm for 30 min.
4. Subsequently the extract is left to stand for 15 min.
5. Due to clogging of the normally used Whatmann filters, the soy extracts are filtered over a nylon sieve (pore size 20 micron).
6. Filtrates are added to CuCl$_2$ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 80-230 (subst-CuCl$_2$ in mg or µl); see figure 11.1.1.

![Figure 11.1.1. Concentration ratio 80-230 (2006.06.15.CK, 26-02, 12h29). Sample Soy #53](image)

11.2 Research Questions

*Can different production systems (organic versus conventional) be discriminated?*

This was assessed in the project ‘Organic more healthy?’; experiments 2006.06.15.CK and 2006.06.20.CL. Two samples originating from an organic and conventional production system could be differentiated highly significantly by textural image analysis and ‘Two group testing’.
Of course the results from this pilot only reflect the difference between these two individual samples (analytical data available!). These results do not serve as a solid basis for the discrimination between organically and conventional produced soy in general.

*Can FIBL DOK soy samples (organic versus conventional) be discriminated?*

This was assessed in the same trial as above. Differentiation was possible with both textural analysis and ‘Two group testing’. Samples were analysed on two separate days, see figure 11.2.1.

![Figure 11.2.1. Photos from experiments 2006.06.15.CK and 2006.06.20.CL, demonstrating the effect of different production systems (DOK samples below).](image-url)
12 TRITICALE SAMPLE PREPARATION

12.1 Procedure

1. The samples are cleaned by means of a 2.0mm Retsch sieve, followed by manually removing of damaged and broken kernels and remaining foreign particles.
2. 100g of each sample is grinded with a centrifugal mill (Retsch ZM 100; 14.000 rpm) fitted with a 1.0 mm ring sieve.
3. 50.0g of the milled material is extracted in 450.0 ml deionised water (25°C) on a (Heidolph Unimax 2010) horizontal shaker at 200 rpm for 30 min.
4. Subsequently the extract is left to stand for 15 min.
5. 50 ml extract is filtered over respectively Whatmann 41 and 40 paper filters. The total (dual) extraction time is set at 45 minutes.
6. Filtrates are added to CuCl$_2$ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 70-130 (subst-CuCl$_2$ in mg or µl); see figure 12.1.1.

![Figure 12.1.1. Concentration ratio 70-130 (2006.06.23.CM, 05-05, 13h31) Triticale #62](image)

12.2 Research Questions

*Can different production systems (organic versus conventional) be discriminated?*

This was assessed in the project ‘Organic more healthy?’; experiments 2006.06.23.CM and 2006.06.29.CN. Two samples originating from an organic and conventional production system could be differentiated significantly by textural image analysis and ‘Two group testing’, see figure 12.2.1.
Of course the results from this pilot only reflect the difference between these two individual samples (analytical data available!). These results do not serve as a solid basis for the discrimination between organically and conventional produced triticale in general.

Figure 12.2.1. Photo from experiments 2006.06.23.CM, demonstrating the effect of different production systems.
13  MAIZE SAMPLE PREPARATION

13.1  Procedure

1. The samples are cleaned by means of a 2.0mm Retsch sieve, followed by manually removing of damaged and broken kernels and remaining foreign particles.
2. 100g of each sample is grinded with a centrifugal mill (Retsch ZM 100; 14.000 rpm) fitted with a 1.0 mm ring sieve.
3. 50.0g of the milled material is extracted in 450.0 ml deionised water (25°C) on a (Heidolph Unimax 2010) horizontal shaker at 200 rpm for 30 min.
4. Subsequently the extract is left to stand for 15 min.
5. 50 ml extract is filtered over respectively Whatmann 41 and 40 paper filters. The total (dual) extraction time is set at 45 minutes.
6. Filtrates are added to CuCl$_2$ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 190-120 (subst-CuCl$_2$ in mg or µl); see figure 13.1.1.

![Figure 13.1.1. Concentration ratio 190-120 (2006.05.05.BY, 31-01, 13h20) Maize #54](image)

13.2  Research Questions

*Can different production systems (organic versus conventional) be discriminated?*

This was assessed in the project ‘Organic more healthy?’; experiments 2006.05.05.BY and 2006.05.10.BZ.Two samples originating from an organic and conventional production system could be differentiated highly significantly by textural image analysis and ‘Two group testing’, see figure 13.2.1.
Of course the results from this pilot only reflect the difference between these two individual samples (analytical data available!). These results do not serve as a solid basis for the discrimination between organically and conventional produced maize in general.

Figure 13.2.1. Photo from experiments 2006.05.05.BY, demonstrating the effect of different production systems.
14 BARLEY SAMPLE PREPARATION

14.1 Procedure

1. The samples are cleaned by means of a 2.0mm Retsch sieve, followed by manually removing of damaged and broken kernels and remaining foreign particles.
2. 100g of each sample is grinded with a centrifugal mill (Retsch ZM 100; 14.000 rpm) fitted with a 1.0 mm ring sieve.
3. 50.0g of the milled material is extracted in 450.0 ml deionised water (25°C) on a (Heidolph Unimax 2010) horizontal shaker at 200 rpm for 30 min.
4. Subsequently the extract is left to stand for 15 min.
5. 50 ml extract is filtered over respectively Whatmann 41 and 40 paper filters. The total (dual) extraction time is set at 45 minutes.
6. Filtrates are added to CuCl$_2$ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 80-240 (subst-CuCl$_2$ in mg or µl); see figure 14.1.1.

![Figure 14.1.1. Concentration ratio 80-240 (2006.05.19.CC, 01-01, 13h10) Barley #58](image)

14.2 Research Questions

*Can different production systems (organic versus conventional) be discriminated?*

This was assessed in the project ‘Organic more healthy?'; experiments 2006.05.19.CC and 2006.05.22.CD. Two samples originating from an organic and conventional production system could be differentiated significantly by textural image analysis. ‘Two group testing’ gave no significant differentiation, see figure 14.2.1.
Of course the results from this pilot only reflect the difference between these two individual samples (analytical data available!). These results do not serve as a solid basis for the discrimination between organically and conventional produced barley in general.

Figure 14.2.1. Photo from experiments 2006.05.19.CC, demonstrating the effect of different production systems.
15 RYE SAMPLE PREPARATION

15.1 Procedure

1. The samples are cleaned by means of a 2.0 mm Retsch sieve, followed by manually removing of damaged and broken kernels and remaining foreign particles.

2. 100g of each sample is grinded with a centrifugal mill (Retsch ZM 100; 14.000 rpm) fitted with a 1.0 mm ring sieve.

3. 50.0g of the milled material is extracted in 450.0 ml deionised water (25°C) on a (Heidolph Unimax 2010) horizontal shaker at 200 rpm for 30 min.

4. Because the extract clogs up the commonly used Whatmann filters, 15 ml extract is centrifuged at 4000 rpm (2940g) for 15 min.

5. Extracts are added to CuCl$_2$ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratios yielding pictures with a well defined ramification structure are 85-250 and 100-250 (subst-CuCl$_2$ in mg or µl); see figure 15.1.1.

![Figure 15.1.1. Concentration ratios 85-250 (left, 2008.04.16.FO, 01-01, 11h09) and 100-250 (right, 2008.04.16.FO, 33-03, 10h38)](image)

15.2 Research Questions

*Can different production systems (organic versus conventional) be discriminated?*

This was assessed in the project ‘Results rye comparison London and Scottish, 2008’; experiment 2008.04.16.FO. Two samples originating from an organic and conventional production system could be differentiated by textural image analysis and characterized by conventional profiling, see figure 15.2.1.
Of course the results from this pilot only reflect the difference between these two individual samples (analytical data available!). These results do not serve as a solid basis for the discrimination between organically and conventional produced rye in general.

Figure 15.2.1. Photos from experiment 2006.05.05.BY, demonstrating the effect of different production systems.
16 GOAT MILK SAMPLE PREPARATION

16.1 Procedure

1. The bottles containing the milk samples were manually swirled gently in vertical position for 2-3 times to collect the cream debris onto the bottle rim.
2. To homogenise the samples, the bottles were swirled vigorously by hand for approximately 10 seconds, after which they were shaken by turning upside-down, whilst turning the bottle, 10 times.
3. This procedure was carried out twice after which a 50ml sample was poured into a 100ml glass beaker and placed into a 20 degrees waterbath for 30 minutes.
4. The milk sample was added to CuCl₂ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 400-250 (subst-CuCl₂ in mg or µl); see figure 16.1.1.

![Figure 16.1.1. Concentration ratio 400-250 (2008.11.12.GE, 33-01 goat sample #168, 12h32)](image)

16.2 Research Questions

*Can goats, characterized on the basis of threefoldness, be differentiated correctly with the crystallisation method?*

This was assessed in the report ‘Geitenmelk studie, November-december 2008’. Differentiation on the basis of conventional profiling and textural image analysis could not identify the alleged threefolded typicity of the goats. However a correlation was found with the protein content of the milk samples.

*Does the pipetting order of CuCl₂ and goat milk affect the crystallisation image?*
This was assessed in the report ‘Geitenmelk studie, November-december 2008’. No effect was found for the tested concentration ratios, which however do show a strong effect for cow milk crystallisations. This implies that flocculation of cow milk is not due to the protein content alone.

16.3 **Recommendations for future research**

Standardisation on the protein content in the milk might show the more subtle differences.
17 CREAM CHEESE (FRISCHKÄSE) SAMPLE PREPARATION

17.1 Procedure
1. From each Frischkäse sample a 100g sub sample is taken, which is manually homogenized.
2. The water soluble components are obtained via an extraction in 200ml 30°C bi-distillated water (250 rpm; 30 min).
3. Insoluble components are removed from the resulting extract by filtration over a 20-25µm paper filter (whatmann #1441150).
4. The filtrate is added to CuCl$_2$ solutions and shaken 120 rpm for 30 min before pipetting in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 1000-150 (subst-CuCl$_2$ in mg or µl); see figure 17.1.1.

![Figure 17.1.1. Concentration ratio 1000-150 (2007.02.06.DS) #637; Organic](image)

17.2 Research Questions

Can different production systems (organic versus conventional) be discriminated?

This was assessed in the project ‘Biocrystallisation Hochland Milk and Frischkäse, February 2007’; experiment 2007.02.06.DS. Two samples originating from an organic and conventional production system could be differentiated highly significantly by textural image analysis. Visual evaluation ‘conventional profiling’ characterised the organic Frischkäse sample as better due to a better differentiated needle structure. However, the production of the two samples required slight differences in the processing steps which might cause the observed differences in the crystallisation images.
Therefore these results do not serve as a solid basis for the discrimination between organically and conventional produced Frischkäse in general.

17.3 Recommendations for future research

Because the variation in the crystallisation starting time is quite large (>7 hours; SD 1:48h), a higher CuCl2 concentration (300mg) may be required, which is shown to reduce the variation and decrease the tcrStart. This will have to be combined with a more concentrated extraction procedure (1:1; 100g in 100ml).
18  APPLE AND BOTTLED APPLE JUICE SAMPLE PREPARATION

18.1 General Procedure

1. The apples are cleaned and split into segments, thereby omitting the core.
2. One half of the apples is used for juice preparation.
3. The apple half is chosen so that the blush is equally divided on both halves.
4. Juice is produced with the ‘Greenstar’ (Kiemling Naturkost; D) juice extractor, fitted with the light resistance spring.
5. Freshly prepared juice and bottled juices are filtered over a 110 micrometer nylon filter and left to settle for 20 minutes at RT to allow for sedimentation of starch.
6. The dry matter contents of the filtered juices are determined in triplicate by heating overnight at 103°C ± 2 degrees.
7. Filtrates are added to CuCl₂ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratios yielding pictures with a well defined ramification structure are 230-200 (subst-CuCl₂ in mg or µl); 275-200 and 325-200 see figure 18.1.1.

The Greenstar sieve is cleaned after each sample, as the apple skin tends to clog up the sieve.

18.2 Juice degradation procedure

To further broaden the analysis, a degradation step can be performed.

1. The juices are stored at 4°C for 4 days.
2. Prior to the crystallisation, the juices are heated in a microwave oven at 750W for 90° (≈ 70°C) and subsequently filtered over a 110 micron nylon sieve.
18.3 Research Questions

*Can different apple juice processing steps be distinguished?*

This was assessed in the report ‘Processing of apple juice, a pilot study, November-December 2007’; experiments 2007.10.29.EVand 2007.11.01.EW. For this, 1 apple sample and 2 bottled apple juices (turbid pasteurised and clear pasteurised from a concentrate) forming 2 apple juice processing steps, were analysed at three concentration ratios on two days. The first day the juices were analysed untreated, for the second analysis day the juices were degraded in a standardised manner. A clear effect, reflecting the intensity of the processing was found with textural image analysis and could be characterized by visual evaluation ‘conventional profiling’ (see figure18.3.1).

However, the fact that the different processing steps were not performed with the same apple samples should be taken into account as this could explain partly the found differences.
Can different apple varieties be distinguished?
This was assessed and confirmed on the basis of conventional profiling in report ‘Natudis apple study, November-December 2007’. However, no analytical data substantiating this result is available. For the analysis fresh and degraded apple juice was used.

Can different production systems (organic versus conventional) be discriminated?
This was assessed and confirmed on the basis of conventional profiling in report ‘Natudis apple study, November-December 2007’. However, no analytical data substantiating this result is available. For the analysis fresh and degraded apple juice was used.

Can the apple quality be characterized in terms of the Inner Quality aspects Growth, Differentiation and Integration?

Figure 18.3.1. Photos from experiment 2007.10.29.EV, demonstrating the effect of processing of apple juice at concentration ratio 180-125.
This was assessed and partly confirmed on the basis of conventional profiling in report ‘Parameters for Apple Quality-2; 2001 – 2003’.

18.4 Recommendations for future research

Juice extracted from the apple variety ‘Gala’ tends to be too viscous to be filtered. Therefore experimentation with an alternative procedure based on extraction may be worthwhile. Extraction will also reduce the starch concentration in the final filtrate.
19

ONION SAMPLE PREPARATION

19.1 Procedure

1. Samples consist of at least 10 onions. Storage is at RT in the dark.
2. The root tip and the leave node are cut off with a stainless steel knife. The outer (predominantly brown) leaves are completely removed. Onions that have sprouted are omitted from juice preparation.
3. The onions are cut into 4 equal segments. Rotten parts are cut out. Only one half of the onion is used for juice preparation, the other is used to determinate the dry matter.
4. The onion halves are grated with a Zyliss mill assembled with the rough grinder, delivered with the mill.
5. 50.0gr of the milled material (mix grate thoroughly by hand) is transferred into a 500 ml wide-necked Erlenmeyer flask.
6. 450.0 ml of high purity water (25°C) is transferred into the flask while simultaneously manually shaking the flask, thereby securing that all milled material is in contact with the water.
7. The Erlenmeyer is placed in 500 ml flask holders on a shaker (Heidolph Unimax 2010) at 200 rpm for 30 min.
8. The required amount of extract is filtered through a 100µm nylon filter.
9. The filtrate is added to CuCl$_2$ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

Concentration ratios yielding pictures with a well defined ramification structure are 150-100 (subst-CuCl$_2$ in mg or µl) and 250-150.

19.2 Research Questions

No data available
20 CHEESE SAMPLE PREPARATION

20.1 Procedure

1. The cheese is analysed directly out of cold storage (4-7°C).
2. From the inner part of the cheese a sample of approximately 30g is taken.
3. The cheese is manually pressed through a 2.0mm stainless steel Retsch sieve.
4. 20.0g grate is added to a 500ml wide necked Erlenmeyer, taking care that the single grates are not pressed into a clump.
5. 30°C bidest is added up to a total weight of 200.0g.
6. Water soluble components of the cheese are extracted by placing the Erlenmeyer on a Heidolph horizontal shaker (200 rpm for 30 min.).
7. 50g of the extraction fluid is filtrated over a 20µm paper filter (Whatman 41), the total filtration time is 3 minutes.
8. The filtrate is manually swirled to homogenisation (approx. 2 sec.), and added to CuCl₂ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 300-200 (subst-CuCl₂ in mg or µl), see figure 20.1.1.

![Image](image.png)

Figure 20.1.1. Concentration ratio 300-200 (2005.12.19.BI, 16-04, 12h42) sample Rouveen2, conventional.

20.2 Research Questions

Can different production systems (organic versus conventional) be discriminated?

This was assessed, however the production systems could not be differentiated significantly, see report ‘Productkwaliteit zuivel: verschil tussen biologisch en gangbaar (2006); Rapportnummer 23’. However, in this study the results for 5 different cheese varieties (from
an organic and conventional origin) were grouped. Therefore the conclusion seems a little bold.

*Can different varieties/producers be distinguished?*

This was assessed and confirmed by conventional profiling for series 2005.12.19.BI. The producer effect exceeds the organic/conventional origin of the milk, as reflected in the crystallisation pictures, see figure 20.2.1.

![Figure 20.2.1. Photos from experiment 2005.12.19.BI, demonstrating the effect of different cheese varieties.](image-url)
21 WHEAT SAMPLE PREPARATION

21.1 Procedure

The sample can be stored cleaned as well as un-cleaned. After receipt the sample is stored in labelled air-tight plastic bags approved for foodstuffs. Short-term storage (< 30 days) takes place in a dark room at 6°C. Long-term storage (> 30 days) takes place in a room with temperature and humidity control at 2-3°C and 40-50% rH, with protection against insects and rodents. 24 h before start of examination the sample is placed at RT, avoiding exposure to direct sunlight.

1. After thoroughly mixing the sample, 240g is taken out for sample preparation.
2. The sample is cleaned by means of a 2.0 mm sieve, followed by a manual cleaning procedure, whereby damaged and broken kernels and foreign seeds and particles are removed.
3. Each sample is crystallised in replicate (two times sample preparation); replicates consisting of 100gr cleaned kernels each.
4. A centrifugal mill (Retsch ZM 100; 1.0 mm ring sieve) is applied for grinding. Settings 14.000 rpm; load control ≤ 4; total grinding time ≈ 60”.
5. After milling the sample, the flour is transferred to a glass bowl.
6. 50.0g of the milled material is transferred into a 500 ml wide-necked Erlenmeyer flask. 450.0 ml of high purity water (25°C) is successively transferred into the flask while simultaneously manually shaking the flask, thereby securing that all milled material is in contact with the water.
7. Extraction is performed on a horizontal shaker (Heidolph Unimax 2010) at 200 rpm; 30’.
8. Subsequently the extract is left to stand for 15 min.
9. 50 ml extract is pipetted from the settled extract at 2 cm below the surface level, and filtrated over respectively Whatmann 41 and 40 paper filters placed in a glass or plastic funnel, which is placed into a narrow-necked 100 or 250 ml Erlenmeyer flask.
10. Manually swirl the filtrate to homogenisation (approx. 2 sec.) between the two filtration steps.
11. The total (dual) extraction time is 45 minutes.
12. The final filtrate is manually swirled to homogenisation (approx. 2 sec.) and added to CuCl₂ solutions and pipetted in 5-6 fold replicate in the crystallisation chamber. 30’; 100rpm.

The concentration ratios yielding pictures with a well defined ramification structure are 90-90 (subst-CuCl₂ in mg or µl); 70-90 and 110-90, see figure 21.1.1.

Before milling the next sample, the sieve and the rotating parts are cleaned, by means of a soft brush and a vacuum cleaner. The stainless steel parts are rinsed with hot water/bidest. Take care to remove any kernels that may be jammed in the inlet hole of the Retsch mill.

Sample preparation with replicates is performed in the following way: A1B1C1C2B2A2 (sample and replicate performed the same day). In the case the replicates are performed another day, sample preparation of the replicates is again in the reversed order (C2B2A2).
21.2 Research Questions

*Can different varieties be discriminated?*

This was assessed in the article Doesburg and Huber (2007); experiment 2007.04.04.DT. Three samples consisting out of three coded subsamples each could be grouped correctly by visual evaluation ‘Grouping’. Textural image analysis could differentiate significantly 2 out of 3 samples.

*Can different production systems (organic versus conventional) be discriminated?*

This was assessed in the project ‘Organic more healthy?’; experiments 2006.08.04.CQ, 2006.08.07.CR and 2006.08.08.CS. Two samples originating from an organic and conventional production system could be differentiated significantly by textural image analysis, and highly significant by ‘Two group testing’. Moreover, the textural image analysis results could be confirmed by the BRAD and Uni Kassel laboratories. Of course the results from this pilot only reflect the difference between these two individual samples (analytical data available!). These results do not serve as a solid basis for the discrimination between organically and conventional produced wheat in general.
22 MILK SAMPLE PREPARATION

22.1 Procedure

Note: the pipetting order of CuCl$_2$ and water remains under investigation!

1. Slowly swirl the bottle containing the milk sample 2-3 times, whilst standing up, to collect the cream debris on the bottle rim.
2. Gently shake the bottle 10 times up and down whilst turning the bottle between each ‘shake’.
3. Swirl the bottle for approximately 10”
4. Finally, shake the bottle again 10 times up and down whilst turning the bottle between each ‘shake’.
5. Pour approximately 50ml into a 100ml bekerglass.
6. Place the bekerglass into a 20 degrees waterbath for 30 minutes
13. Manually swirl the heated milk sample to homogenisation (approx. 2 sec.) and add to the CuCl$_2$ solutions.
14. Pipette in 5-6 fold replicate in the crystallisation chamber.

The concentration ratios yielding pictures with a well defined ramification structure are 200-150 (subst-CuCl$_2$ in mg or µl) and 325-250, see figure 22.1.1.

Figure 22.1.1. Concentration ratio 200-150 (2006.12.01.DM, 07-07, 13h31) sample raw milk.

22.2 Research Questions

Can the effect of processing (homogenisation/pasteurisation) be discriminated and characterized?
This was assessed and confirmed in the report ‘Biocrystallisations, milk treatments, February 2007’ and partly published in Kahl (2009). The pipetting order was ‘milk to (CuCl$_2$ to water)’. By means of Visual evaluation, 5 groups of treatment could be differentiated out of the 6 (raw and 5 treatments) groups. The treatments were: homogenisation at 50Bar; homogenisation at 200Bar; homogenisation at 200Bar and subsequently pasteurisation at 76$^\circ$C; homogenisation at 200Bar and subsequently pasteurisation at 90$^\circ$C and ultra high temperature sterilisation (UHT) without homogenisation at 140$^\circ$C. No clear distinction was possible between the homogenised samples (200 bars) with subsequent heating at 76$^\circ$C or 90$^\circ$C. Textural image analysis could significantly differentiate the crystallisations originating from the 200Bar homogenised, the UHT sterilised and the two combined homogenisation and pasteurisation treatments (76$^\circ$C and 90$^\circ$C) from all other treatments.

**Does the pipetting order effect the crystallisation picture?**

Contrary to goat milk, the pipetting order has a dramatic effect on the crystallisation picture, see figure 22.2.1. As the pipetting order has no effect on goat milk crystallisations, this implies that flocculation of cow milk is not due to the protein content alone. Apparently the correct pipetting order is CuCl$_2$ to (milk to water)! See figure 22.2.1.

![Figure 22.2.1. Photos from experiment 2006.10.19/DD, demonstrating the effect of the different pipetting order on a raw milk sample.](image)

**Can different production systems (organic versus conventional) be discriminated?**

This was assessed and confirmed in the reports ‘Summary - Organic products and health - Results of milk research 2005’ by Slaghuis and de Wit (2006). The pipetting order was ‘milk to (CuCl$_2$ to water)’. Discrimination was obtained by visual evaluation ‘Conventional profiling’ and a prototype of the structural image analysis software. Both studies showed that ‘organic milk is systematically more ‘ordered’: it has a more ‘ordered structure’ and better ‘integration and coordination’. 
Are cow age differences reflected in the milk crystallisations?

This was mainly assessed in experiment 2004.10.12.MC and report ‘Melk kristallisaties april – oktober 2004’. No correlation was found with the cow age, however a strong correlation was found with the cow breed (MRIJ or HF), and to a slightly lesser extent to the protein content.

22.1 Recommendations for future research

- Standardisation on the protein content in the milk, this may show more subtle differences.
- Further work on the effect of cow age after extensive standardisation on breed and protein content!
CARROT SAMPLE PREPARATION

23.1 Procedure

Samples consisting of at least 60 preferably standardised (length and diameter) carrots are taken, aberrant carrots are not included. Ideal storage conditions are 5°C, 98% rH. Although fresh carrots are preferred, maximal storage time is 1 week for 'summer-carrots' and 7 months for 'winter-carrots' (leaves removed).

1. The carrots are cleaned in a bucket with a soft sponge under slowly running tap-water until all sand and debris is removed. In this way the debris will sediment in the bucket, allowing an easy disposal.
2. The cleaned carrots are dried by padding them with a clean paper towel.
3. The head and the tail of the un-peeled carrots are excised, leaving approximately 2/3 for juice preparation.
4. Side roots and damages by carrot pests are cut out.
5. All carrots are vertically cut into two equal parts with a sharp stainless steel knife. One of the halves is halved again, producing two quarters. One quarter is used for sample preparation. The other to clean the Greenstar in between different samples.
6. Proceed to this step for all carrot samples before juice is prepared.
7. Juice is produced with the Green Star GS3000 juice extractor fitted with the smallest sieve and the valve with the light resistance spring. The spring is depressed a couple of times before use!
8. The spring is screwed on until it reaches its resisting point.
9. Before juice is collected from each carrot sample, first process the excess carrot quarters of this sample until the Green star is saturated and juice is produced.
10. Filter the juice with a 110 micrometer nylon filter placed in a glass funnel. To lower the extent of the oxidation, place the end of the funnel tube against the cylinder.
11. Cover the measuring cylinder with parafilm and let the juice settle for 20 minutes at RT to allow for sedimentation of starch.
12. The settled juice in the measuring cylinder is directly added after the 20 minutes settling period to 100ml wide-necked Erlenmeyers already containing H2O and CuCl2, to prepare the necessary chamber solutions. For this the juice is pipetted approximately 2 cm under the surface level (probably the oxidated area) of the measuring cylinder.

The concentration ratios yielding pictures with a well defined ramification structure are 115-90 (subst-CuCl2 in mg or µl) and 190-150, see figure 23.1.1.

When the replicates are performed on the same day, juice preparation is according to the following schedule: sample A1B1C1C2B2A2. In the case the replicates are performed another day, sample preparation of the replicates is again in the reversed order (C2B2A2).
23.2 Research Questions

*Can different varieties be discriminated?*

This was assessed in the report ‘Kwaliteitsonderzoek Nautilus B-peen; rassenproef 2003-2004’. The different varieties could be discriminated and a quality interpretation was performed according to the Inner Quality Concept.

Figure 23.1.1. Concentration ratio 115-90 Fleck Möhre.
24 PVP SAMPLE PREPARATION

24.1 Procedure

1. 0.400 g polyvinyl pyrrolidone (C₆H₉NO)n is transferred into a 500ml wide necked Erlenmeyer, containing 100ml distilled water, placed on a balance.
2. Distilled water is added to 400.00 g.
3. The flask is placed on a horizontal shaker at settings 200rpm; 30’ (no visible surface turbulence should be visible!).
4. Check for the presence of non-dissolved PVP-particles, if so, place the flask for an additional 30’ on the shaker.

The concentration ratio yielding pictures with a well defined ramification structure is (substituting CuCl₂ in mg or µl).
### 25 Freeze Dried Wheat Meal, Chamber Standard Sample Preparation

#### 25.1 Procedure

The chamber standard is pipetted in 6-7 fold repetition in each crystallisation experiment, thereby covering the two sensor cells in the crystallisation apparatus. To calibrate the chamber (determine the 50% evaporation time), two times sample preparation is performed, after which the final extracts are pooled and pipetted into the 43 locations in the crystallisation chamber.

1. 50.0g meal is transferred into a 500 ml wide-necked Erlenmeyer flask. 450.0 ml of high purity water (25°C) is successively transferred into the flask while simultaneously manually shaking the flask, thereby securing that all meal is in contact with the water.
2. Extraction is performed on a horizontal shaker (Heidolph Unimax 2010) at 200 rpm; 30'.
3. Subsequently the extract is left to stand for 15 min.
4. 50 ml extract is pipetted from the settled extract at 2 cm below the surface level, and filtrated over respectively Whatmann 41 and 40 paper filters placed in a glass or plastic funnel, which is placed into a narrow-necked 100 or 250 ml Erlenmeyer flask.
5. Manually swirl the filtrate to homogenisation (approx. 2 sec.) between the two filtration steps.
6. The total (dual) extraction time is 45 minutes.
7. The final filtrate is manually swirled to homogenisation (approx. 2 sec.) and added to CuCl\(_2\) solutions and pipetted in 6-7 fold replicate in the crystallisation chamber.

The concentration ratio yielding pictures with a well defined ramification structure is 90-90 (subst-CuCl\(_2\) in mg or µl).
REFERENCES


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