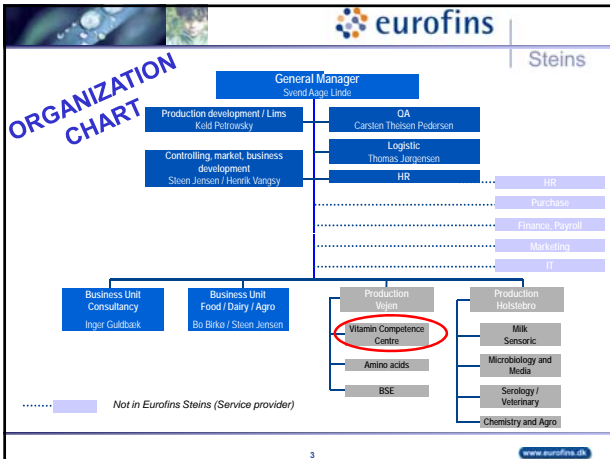


Vitamin Competence Centre

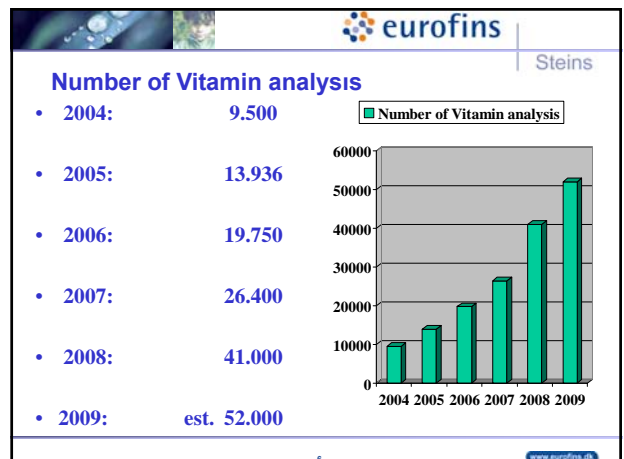
Region Syd
 Palle B. Sørensen
 Per K. B. Nilsson

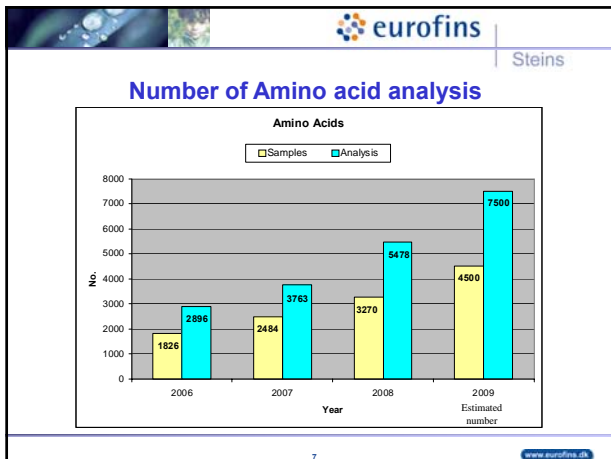
Holstebro: Dairy product, common chemical & microbiological analysis for food & feed
 Galten: Product testing, environmental analysis
 Vallensbæk: Pharma
 Vejen: Environmental & Vitamin analysis: Ladelundvej 85 6600 Vejen, Danmark.



Capacity of Vitamin Competence Centre

- 2 chemists
- 3 customer service
- 20 lab technicians – 3 in nightshift
- 17 HPLC lines
- 2 LC-MS lines
- 1 GC-MS
- Ion-chromatograph
- 1 automated SPE
- 2 Biacore
- 2 automated Gilson diluters
- 2 automated Gilson for measuring microvitamins
- 4 Amino Acid lines (Biochrom)
- Chromleon Software platform





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- ## Vitamins
- Stability
 - Precautions and considerations
 - Methods in general
 - Quality assurance
 - Other analysis than Vitamins
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Characteristics of Vitamins (stability)

Vitamins	Moist	Heat	Light	Oxidation	Reduction	with metal
Retinols (A)	+	++	++	++	0	++
Cholecalciferol (D3)	+	++	++	+	0	++
Tocopherols (E)	+	+	++	++	0	++
Menadione (K1)	+	++	+	0	++	++
Thiamine (B1)	+	++	0	+	++	++
Riboflavin (B2)	0	0	++	0	++	0
Niacin (B3)	0	0	0	0	0	+
Pantothenic acid (B5)	++	+	0	0	0	0
Pyridoxine (B6)	0	0	+	0	0	++
Biotin (B8)	0	+	0	0	0	0
Folic acid (B9)	+	++	++	0	0	+
Cyanocobalamin (B12)	0	+	+	+	+	+
Ascorbic acid (C)	+	0	++	++	0	++

++ = very sensitive, + = sensitive, 0 = stable.

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- ### Precautions during analysis
- UV-protected light and windows in the lab
 - Cold/hot saponification to extract the vitamins
 - Adding antioxidants during the analytically process
 - Using nitrogen to remove oxygen
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- ### General methods for vitamins
- #### Fat-soluble vitamins (food/feed)
- Saponification and extraction with ether or hexane followed by HPLC analysis and detection by uv-abs or emission
 - A, E, E-profile, K1 and beta-carotene
 - D2 & D3 done by HPLC-MS
 - K3 (MSB) done by GC-MS
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- ### General methods for vitamins
- #### Water-soluble vitamins (food/feed)
- Enzymatic treatment followed by HPLC (B1+B2+B6+C)
 - Microbiological analysis (B3+B5+B8+B9+B12)
 - Ionchromatography (Choline)
 - Biacore (B8+B12+B9)
- #### USP (premixes & supplements and tablets)
- Extraction using various solvents followed by HPLC analysis and detection by UV-abs or fluorescence
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AA / EE -vitamins

- A: Sum of all trans-retinol +13-cis-retinol
- E: α -tocopherol/ α - β - γ - δ -tocopherol (E-profile)
- Hot Saponification (KOH) \rightarrow Extraction (hexan/ethylacetate) \rightarrow rp-HPLC-FLD/DAD

β -carotene

- β -carotene are reported as trans- β -carotene
- Cold Saponification \rightarrow Extraction (hexan/tetrahydrofuran) \rightarrow rp-HPLC-VIS/DAD. For fruit, vegetables and juices.
- Hot saponification \rightarrow Extraction (hexan/tetrahydrofuran) \rightarrow rp-HPLC-VIS/DAD. For fatty samples

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DD-2/ DD-3 vitamins

- D2: Ergocalciferol
- D3: Cholecalciferol
- Enzymatic treatment (Pancreatin) \rightarrow Hot Saponification (KOH) \rightarrow Extraction (ether) \rightarrow Cleaning-up (SPE column n-heptane/diethyl ether) \rightarrow np-HPLC-UV/DAD (fraction collection) \rightarrow rp-HPLC- UV/DAD/MS

KK-1/ KK-3 vitamins

- K1: Phylloquinone and Phytonadione
- K3: MSB (Menadione Sodium Bisulfite)
- K1: Enzymatic treatment (Lipase) \rightarrow Extraction (ether) \rightarrow rp-HPLC-FLD
- K3: Extraction (dichloromethane) \rightarrow GC-MS

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- Vitamin D (RDI = 5 μ g)
 - Uptake of calcium and phosphor for bones and teeth
 - Fetus development
 - Buildup of tissue and neural function
 - Immunity system
- Vitamin A (RDI = 800 μ g)
 - Deficiency causes irreversible blindness
 - Regulating insulin production
 - Development of pancreatic cells
- Vitamin E (RDI = 10 mg)
 - Acting as antioxidant and believed to prevent cancer and cardiovascular diseases
- Vitamin K (RDI = 70 μ g)
 - Important factor for the coagulation of blood

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- Source of Vitamin D:
 - UV-light
 - Eggs and fish oil (D3)
 - Plant oil (D2)
 - Fortified food (D2 or D3)
- Provitamin D \leftrightarrow Previtamin D \leftrightarrow Vitamin D
- Stability
 - Light and acid condition can cause isomerization from vitamin D to previtamin D.

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- Sources of vitamin A:
 - Fish, meat, eggs and dairy products (animal kingdom)
- Sources of Betacarotene (provitamin A):
 - Fruits, carrots and dark green leafy vegetables (plant kingdom)
- Synthetic vitamin A commonly used for fortified products:
 - Acetat- and palmitat esters of all-trans-retinols

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- Vitamin A is measured as the content of all-trans-retinol and 13-cis-retinol.
- Vitamin A is reported as μ g or IU (1 IU = 0,3 μ g retinol)
- Betacaroten is reported as trans-betacarotene.
- Retinol equivalent (RE)/provitamin A
 - 1 RE = 1 μ g retinol = 6 μ g beta carotene
- Different values for different forms of vitamin A
 - All-trans-retinol acetate = retinol x 1,147
 - All-trans-retinol palmitate = retinol x 1,833
- What kind of vitamin has been added by the customer?

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- Sources of vitamin E
 - Plant oil – nuts – fish – eggs
- Wide range of synthetic forms of vitamin E (acetate/succinate)
- Vitamin E acts as a natural antioxidant and is used as such in the food industry and reacts with free radicals and oxygen
- Light, heat and various metals in combination with oxygen can lead to rapid loss of vitamin E

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- Vitamin E is measured and reported as the content of a-tocopherol (mg/100g)
- Vitamin E profile is reported as the content of δ, β, γ and a-tocopherol (mg/100g)
- a-tocopheryl acetate = a-tocopherol * 1,1

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- Vitamin K is measured and reported as the content of phyloquinone K1.
- Sources of vitamin K1, leafy green vegetables (spinach, broccoli, lettuce), certain legumes (green beans) and vegetable oils.
Added to infant formula.
- For human diets vitamin K1 is only added as the phyloquinone. However for pet foods it can be added as several forms of K3 (menadiione).
- Stable to oxidation and food processing. Unstable to light, reducing agents and alkaline conditions.

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B1/B2 vitamins

- B1: Sum of thiamine and its phosphorylated forms
- B2: Sum of riboflavin and riboflavin-5'-phosphate
- Acid hydrolysis (HCl 0,1 M) → Enzymatic treatment (Takadiastase) → rp-HPLC-FLD

CC vitamin

- Sum of L-ascorbic acid & dehydroascorbic acid
- Extraction (((HPO3)n]), oxidation with spatula, reaction with OPD (to form the chromophor form). HPLC with fluoroscenc-detection.

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B6 vitamin

- Sum of pyridoxine, pyridoxal, pyridoxamine & its phosphorylated forms
- Conversion of all the forms into pyridoxine (C₂H₂O₃-H₂O, FeSO₄-7H₂O, phosphatase, NaBH₄) → rp-HPLC-FLD


B3 vitamin

- Extraction with water at room temperature → Cleaning-up (cation exchange SCX column) → rp-HPLC-UV


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- **Water-soluble vitamins**
 - Vitamin C (ascorbic acid) RDI = 60 mg
 - Vitamin C deficiency causes scurvy
 - Important factor for immune system
 - B1 (thiamine) RDI = 1,4 mg
 - Deficiency causes beriberi - mental disorder, confusion and hallucinosis, psychosis and coma
 - B2 (riboflavin) RDI = 1,6 mg
 - Important for energy, protein and lipid metabolism
 - Growth, nails, hair and skin
 - B6 (pyridoxin) RDI = 2 mg
 - Important for protein synthesis and DNA

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- Vitamin C is found in food/feed/nutritional supplements as L-ascorbic acid, dehydroascorbic acid, ascorbyl palmitate, isoascorbic acid (and stay C).
- Vitamin C (L-ascorbic acid) – primarily from the plant kingdom, e.g Citrus, peppers, tomatoes, potatoes.
- Ascorbyl palmitate – used as antioxidant in the food industry
 - Ascorbic acid form with higher lipid solubility.
- Stay C – used in premix in feed
 - Ascorbyl-2-mono- di- and triphosphate

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- Vitamin C is used as a food additive, antioxidant, browning inhibitor, reducing agent, flavour stabilizer, colour stabilizer.
- Vitamin C stability is depending on oxygen, temperature, water, light, metal catalysis, pH (4-6).
- Vitamin C is reported as a sum of all forms in mg/100g.

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- Vitamin B1 exists as free thiamin or phosphorylated thiamin (TMP, TPP, TTP). All forms exist in plant and animal tissues. Free thiamin is found in higher level in plants than animals.
- Major sources of vitamin B1 are unrefined cereal grain, legumes, nuts and pork meat.
- Thiamine hydrochloride (soluble products) and thiamine-mono-nitrate (dry blends) are often used in pharmaceuticals and for food fortification.
- Vitamin B1 is the least stable water soluble vitamin, its highly unstable at alkaline pH.
Baking powder, chocolate products, sulfite (browning inhibitor).

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- All forms are converted into thiamin.
- Thiamine is reported as the sum of thiamine including its phosphorylated derivatives (mono-di-pyro- and triphosphate), mg/100g.
- Factors:
 - Thiamine HCL = Thiamine x 1,27
 - Thiamine-mono-nitrate = Thiamine x 1,23

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- Vitamin B2 in food/feed/supplements exists as riboflavin, riboflavin-5-phosphate
- Good sources is animal products, e.g milk and dairy products. Cereals are low in contents unless fortified.
- Riboflavin and riboflavin-5-phosphate differs greatly in water solubility.
- Vitamin B2 is stable to heat and oxidation if protected to light. Stability increase as pH decreases. pH optimum is 2-5.

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- Riboflavin-5-phosphate is converted to riboflavin during acidic hydrolysis.
- Vitamin B2 is quantified as riboflavin, mg/100g.

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- Vitamin B6 refers to pyridoxal, pyridoxine, pyridoxamine and their phosphorylated derivatives.
- Good sources are meat, cereals, nuts and vegetables.
- Pyridoxine hydrochloride is the synthetic form used for fortification.
- B6 is highly sensitive to light and heat – some compounds are more sensitive than others
- The method converts all forms into pyridoxine, mg/100g.
- B6-Hydrochloride = B6 (pyridoxine) x 1,22

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Risk in HPLC analysis

Overloaded sample

Diluted sample

No.	Ret. Time min	Peak Name	Area mV*min	Amount µg/g
1	12.02	B1-vitamin	2007.611	963.371

HPLC methods – normally 1-2 decades (otherwise risk for overloads)

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Microbiological methods

BB-3 / BB-5 / BB-8 / BB-9 / BB-12 vitamins

- B3: Nicotinic acid & Nicotinamide
- B5: Pantothenic acid
- B8: Biotin
- B9: Total folate / folic acid
- B12: Sum of cyano-, hydroxycobalamin & other cobalamins with vitaminic effect

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Niacin (vitamin B3) by microbiological assay

• Coenzyme NAD

prENmethod will give comparable results depending on extraction step.

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Pantothenic acid (vitamin B5) by microbiological assay

- Our method only determines free, water soluble pantothenic acid
- LC should be possible, but prEN-method not yet ready.

CC(O)(C)C(O)C(=O)NCC(=O)O

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Total biotin by microbiological assay

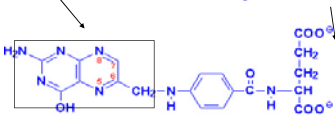
- +6-10 related compounds
- + all of these bound covalently to amino acids
- Bound compounds are liberated with 3 N H₂SO₄
 - LC will be difficult
 - Biacore only free biotin

biotin

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Total folate by microbiological assay

App. 10 related compounds Conjugated with n glutamates



Folic by LC difficult
On Biacore: free folic acid

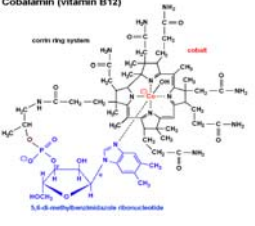
COO⁻
|
CH₂
|
CH
|
COO⁻

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Vitamin B12 by microbiological assay

- ✓ Determines a number of active compounds with varying response
- ÷ False positive response in algae!
- LC difficult: A number of compounds-very low contents. Needs specific clean-up+LC-MS
- Possible with Biacore. Comparable results.

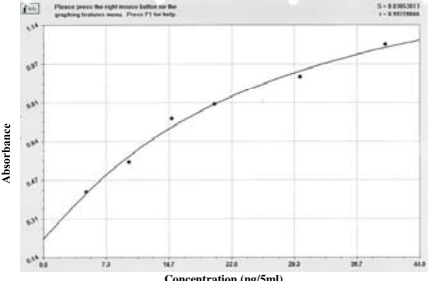
Cobalamin (vitamin B12)



5,6-dimethylbenzimidazole ribonucleotide

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Microbiological methods are based on the growth rate of a microorganism regulated by the vitamin content. Growth is normally measured directly with a turbidimeter.



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METHOD:

- Vitamins are extracted with a specific buffer solution and heated at 121°C for 10 minutes
- Inoculation of the culture
- Incubation at 37 °C for 16-20h
- Turbidity is measured with a spectrophotometer at 650 nm

	Extraction buffer	Microorganism
B3	H ₂ SO ₄ 0,5 M	<i>Lactobacillus plantarum</i>
B5	CH ₃ CO ₂ Na/CH ₃ COOH	<i>Lactobacillus plantarum</i>
B8	H ₂ SO ₄ 3 M	<i>Lactobacillus plantarum</i>
B9	KH ₂ PO ₄ /K ₂ HPO ₄ /C ₆ H ₈ O ₆	<i>Lactobacillus rhamnosus</i>
B12	Na ₂ HPO ₄ /C ₆ H ₈ O ₇ ·H ₂ O	<i>Lactobacillus delbrueckii</i>

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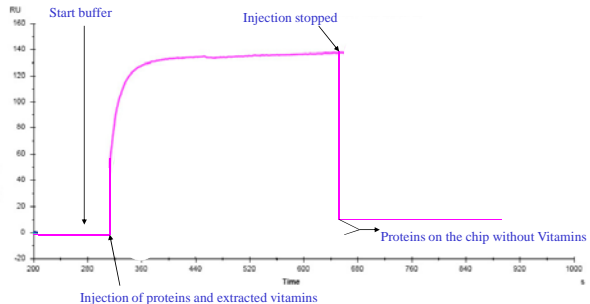
Biacore method



Detection unit

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Removal of Protein – vitamin molecules

● = vitamin ● = protein Y = Surface immobile molecules

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Quality Assurance

- The quality system: DS EN ISO IEC 17025
- Daily control including use of reference materials
- Participation in proficiency test (FAPAS, BIPEA, Nestle, NIST, AAFCO)
- Participation in certification of reference materials
- Comparison with other labs
- Participation in Homogeneity and stability studies (IRMM Validated Supplier).

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Technical Quality

1. Three point calibration (HPLC), seven point calibration (microbiology), QC-samples, reference materials and Control Charts.
2. Verifications will be done when we have reason to believe something is not correct (expected values)
3. Input from quality data
 - Monthly evaluation of all methods (QC-standards and reference materials)
 - Results from proficiency tests, IRMM and NIST project
4. Input from customers (trials, discussions and complaints)

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Collaborative partners

- Nordic Committee on Food Analysis (NMKL)
- Cen/TC 275 WG 9 Vitamins
- Danish Standard Association DS 251 Food, feed, etc.
- Institute for Reference Material and Measurements (IRMM)(Validated supplier Homogeneity and stability studies)
- National Institute of Standards and Technology NIST
- Nestlé Vitamin Centre in Holland
- Danish Institute for Food and Veterinary Research
- Eurofins Wolverhampton
- FAPAS
- Eurofins US Des Moines

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Other analysis

- Amino acids
- Biogenic amines
- Antioxidants
- Miscellaneous

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- Amino acids test

Accredited methods for food & feed

- Acid hydrolysis (except Tryptophane) – DI004
Arginine, Glutamic acid, Phenylalanine, Proline, Threonine, Lysine, Histidine, Leucine, Tyrosine, Ornithine, Aspartic acid, Serine, Glycine, Alanine, Valine, Isoleucine
- Oxidative hydrolysis (except Tryptophane) – DI005
Arginine, Glutamic acid, Phenylalanine, Proline, Threonine, Lysine, Methionine, Cystine, Histidine, Leucine, Ornithine, Aspartic acid, Serine, Glycine, Alanine, Valine, Isoleucine

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Accredited methods for food & feed

- Amino acids tests
 - Tryptophane HPLC – DJ009
 - Multiparameter tests
 - Cystine, Methionine, Lysine & Threonine – DJ014
 - Cystine, Methionine & Lysine – DJ013
 - Cystine & Methionine – DJ011
 - Pure Amino acids – DJA50 (Methionine)
 - DJA51 (Threonine)
 - DJA52 (Lysine)
 For purity testing only (Conc. > 90%)

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Accredited methods for food & feed

- Amino acids packages
 - PDJ01 Total amino acids ex. Tryptophane
 - PDJ02 Total amino acids incl. Tryptophane
 - Free amino acids – PDJ05/ PDJ09 (incl. Taurine)
 - Cystine, Histidine, Lysine, Arginine, Methionine, Tryptophane, Valine, Proline, Glutamic acid, Aspartic acid, Threonine, Serine, Glycine, Alanine, Isoleucine, Leucine, Tyrosine, Phenylalanine, Taurine

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- Biogenic Amines profile (Dansyl) rp-HPLC-UV – DJ700
 - Tyramine, Putresine, Cadaverine, Histamine, Phenylethylamin, Tryptamine, Spermidine, Spermine
 - Cadaverine Dansyl – DJ701
 - Histamine Dansyl – DJ702
 - Biogenic Amines – DJ078 (only together with DJ700)
- Carotenoids (Astaxanthine, Cantaxanthine, Lutein, Zeaxanthin) np-HPLC-vis
- Choline by ionchromatography
- Myo-inositol by GC/MS

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- Ubiquinone (Coenzyme 10 – Vitamin Q)
- Antioxidants by HPLC (BHA, BHT, etoxyquin)
- Preservatives (Benzoic acid, Sorbic acid, sorbates and benzoates)
- Coccidiostatica in feed (Monensin, Narasin & Salinomycin)

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Turn Around Time

The ultimate competition parameter

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