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Paediatric formulations of L-arginine for the use in urea cycle disorders

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Abbreviations

A	Projected pellet surface
AC	Adenylyl cyclise
ADRAC	Adverse Drug Reactions Advisory Committee
API	Active pharmaceutical ingredient
AR	Aspect ratio
ARTG	Australian register of therapeutic goods
ASIC	Acid-sensing ion channel
cAMP	Cyclic adenosine monophosphate
cNMP	Cyclic nucleotide monophosphate
cps	Cycle per second
d	Day
d	Pellet diameter
d_d	Dimensionless diameter
d_{eq}	Equivalent diameter
d_{eq50}	Median of all equivalent diameters
d_{max}	Maximum Feret diameter
$d_{90°}$	Feret-diameter perpendicular to the maximum Feret diameter
DSC	Differential Scanning Calorimetry
EMEA	European Medicines Agency
ENaC	Epithelial-type Na^+ channel
EU	European Union
FDA	Food and Drug Administration
FET	Field effect transistor
GMP	Guanosine 5-monophosphate
GPCR	G-protein-coupled receptor
g	Gram

h	Hours
HCL	Hydrochloric acid
HLB	Hydrophilic lipophilic balance
HPMC	Hypromellose, Hydroxypropyl methylcellulose
HPLC	High performance liquid chromatography
ICH	International conference on harmonisation
IMP	Inosine-5-monophosphate
IP3	Inosine-5-monophosphate
kg	Kilogram
kV	Kilovolt
L	Litre
M	Molar
mA	Milli Ampere
MCC	Microcrystalline cellulose
MDEG1	Mammalian degenerin-1 channel
mEq	Milli equivalent
mg	Milligram
mGluR	Metabotropic glutamate receptor
mL	Millilitre
mm	Millimeter
mmol	Millimole
mM	Millimolar
min	Minutes
mRNA	Messenger ribonucleic acid
MRA	Multiple regression analysis
MSG	Monosodium glutamate
MVDA	Multivariate data analysis
m^2	square meter

n	Number of measurements
NAGS	N-acetyl glutamate synthase
P	p-value of statistic (lack of fit)
p.a.	Pro analysis
PCA	Principal component analysis
PDE	Phosphodiesterase
Ph.Eur.	European Pharmacopeia
PLC	Phospholipase C
PLS	Partial least squares analysis
PROP	6-N-propyl-2-thiouracil
RH	relative humidity
rpm	turns per minute
R^2	Coefficient of determination
SD	Standard deviation
TGA	Therapeutic Good Administration
TRC	Taste receptor cell
UCDs	Urea cycle disorders
US	United States
USP	United States Pharmacopeia
UV	Ultraviolet
WHO	World Health Organisation
X_{50}	Median
x_{Number}	Quantile
ε	Porosity
2D	Two dimension
µmol	Micro milli mole
µm	Micrometer
µL	Microlitre
µg	Microgram

A Introduction and aim of the study

1. Introduction

The fact that children are at risk as they are administered inappropriate medicines has been internationally recognised [146]. Often unlicensed and off-label medicines are the only ones available yet which have not been clinically tested for safety, efficacy and quality in this age group. Most medicines administered to adults have a product licence that outlines the particular indication, dose and route of administration for a drug. However, many medicines used for children are not licensed for use in children or are used outside the terms of the product licence ('off label'). This means that the risks or benefits of using a drug in that particular situation have not been examined by the licensing authority. Problems resulting from the absence of suitably adapted medicines for children include inadequate dosing information leading to increased risks of adverse reactions. These include death, ineffective treatment through under dosing, non-availability to children of therapeutic advances and extemporaneous formulations for children, which may be poorly, or inconsistently, bioavailable and of poor quality [147].

Paediatric drug delivery is a compromise between applicability and preference to achieve conveniently, efficacy with safety. Few studies have been specifically performed to survey the use of different dosage forms in children and the requirements, advantages and disadvantages of various routes of administration and their respective dosage forms have still to be refined. The EMEA reflection paper on formulations of choice for the paediatric population, released for consultation in June 2005 [5], provides the only non-evidence-based rough guide and is an excellent working document for paediatric formulators.
In the reflection paper the most appropriate dosage forms for each age group are evaluated. Liquid formulations such as solutions/drops or effervescent dosage forms are regarded as the gold standard, the most convenient method and rated as the dosage form of choice for infants already from their first month of life [5]. Along with a simple and accurate dose delivery device, the dose volume to deliver is easily adaptable to the patient’s weight.

Suspensions can solve some issues around bad taste but solutions generally have better oral acceptability. Organoleptic characteristics of liquid preparations are of great importance but taste is crucial and varies geographically, making global research and development difficult for companies. Flavouring adequately is one issue but novel and efficient taste-masking options are needed to improve compliance issues due to poor palatability. Moreover, formulating liquids involve more excipients which can have an age-related toxicity [150].

Multiparticulate formulations (e.g. granules, pellets) are solid dosage forms of choice in children (preschool, school) age group, whereas common solid oral dosage forms such as tablets or capsules are only rated as dosage form of choice for older school children and adolescents, as the main problem is the difficulty of swallowing [5]. The small multiparticulates can be dosed directly into the mouth of the patient or can be mixed with food or beverages prior to administration. Similar in their acceptability even for smaller children are the orodispersable dosage forms such as orodispersable tablets, lyophilised wafers or novel dosage forms such as fast dissolving films. They are easy in their administration and do not require additional liquids [150].

Within the present studies an orphan drug is a medicinal product developed for the treatment of a rare disease. In Europe, the official definition of a rare disease is a disease affecting less than 5 per 10000 inhabitants, is fatal, or severely debilitating. Currently over 6000 rare diseases are known, which means that several million people are affected all over the world, over 25 million in Europe alone. Today, treatment exists for only 200-300 of these 6000 diseases [148].

Rare diseases are often inherited, meaning that newborns, children, and young adults often are affected. Physicians may never see a patient with a rare disease. For that reason there is always a risk when a baby is born with a rare disease. A correct diagnosis may not be made and appropriate treatment may not be given [149].

There are rare paediatrics diseases, where hardly any licensed drugs are available. Often children are affected by these diseases. The lack of drug formulation appropriate for children is well acknowledged. One of these diseases is the group of urea cycle disorders (UCDs).

This work focuses on L-arginine as a treatment for urea cycle disorders (UCDs). It is a genetic disorder caused by a deficiency of one of the enzymes in the urea cycle which is responsible for removing ammonia from the blood stream. The urea cycle involves a series of biochemical steps in which nitrogen, a waste product of protein metabolism, is removed from the blood and converted to urea. Normally, the urea is transferred into the urine and removed from the body. In urea cycle disorders, the nitrogen accumulates in the form of ammonia, a highly toxic substance, and is not removed from the body [6]. In April 2000, research experts at the urea cycle consensus conference estimated the incidence of the disorders at 1 in 10000 births. This represents a significant increase in case diagnosis in the last two years [2]. L-arginine or its salts (e.g. monohydrochloride) was identified to be an orphan drug [8] and used in acute and long term therapy of UCDs. The basic principles are to give adequate nitrogen intake for growth, to provide L-arginine as a semi-essential amino acid (except in arginase deficiency), and to stimulate alternative pathways of waste nitrogen excretion with L-arginine.

The oral route is the most easy and favourable route for administration of most drugs. The present investigations focuses on developing taste masked oral liquid dosage forms because L-arginine is reported to exhibit umami and bitter taste [96] and this will significantly improve the quality of treatment provided to suffering patients, especially children, and sustained-release multiparticulate dosage form because L-arginine has a short half-life in the human body for long term therapy of UCDs.

2. Aim of the study

The first aim of this study was the formulation of liquid dosage forms with L-arginine HCL and masking the unpleasant taste of the L-arginine HCL. The L-arginine HCL oral solutions are intended for the long-term therapy or treatment of urea cycle disorders to be administered by the oral route. The stability of those formulations under different storage conditions should be investigated.

The second aim of the study was the formulation of multiparticulate sustained-release solid dosage forms because L-arginine has a short half-life in the human body. Give it in the immediate-release dosage forms it will not maintain the concentration of L-arginine in the plasma stable during the day. This will decrease the therapeutic effect and the compliance of the patients, especially when those patients are infants or children the compliance will be more worse, while in the sustained-release dosage forms as multiparticulate, it can improve the compliance of the patients and maintain the concentration of L-arginine in the plasma stable during the day by decreasing the dose frequency to 2 or 3 times per days.

B General Part

1. Therapeutic use of L-Arginine

Arginine is an amino acid found in many foods, including dairy products, meat, poultry and fish. It plays a role in several important mechanisms in the body, including cell division, wound healing, removal of ammonia from the body, immunity to illness and the secretion of important hormones [140].

Arginine is a conditionally non-essential amino acid, meaning most of the time it can be synthesized by the human body, and does not need to be obtained directly through the diet. The biosynthetic pathway however does not produce sufficient arginine, and some must still be consumed through diet. Individuals who have poor nutrition or certain physical conditions may be advised to increase their intake of foods containing arginine [143].

The body also uses arginine to produce nitric oxide, a substance that relaxes blood vessels and also exerts numerous other effects in the body. Based on this, arginine has been proposed as a treatment for various cardiovascular diseases, including congestive heart failure and intermittent claudication, as well as impotence, female sexual dysfunction, interstitial cystitis and many other conditions. Arginine's potential effects on immunity have also created an interest in using it as part of an "immune cocktall" given to severely ill hospitalized patients and also for preventing colds [143].

Normally, the body either gets enough arginine from food or manufactures all it needs from other widely available nutrients. Certain stresses, such as severe burns, infections and injuries can deplete body supply of arginine. For this reason, arginine (combined with other nutrients) is used in a hospital setting to help enhance recovery from severe injury or illness. Arginine is an amino acid that is best known as a growth hormone releaser [143].

Dietary arginine supplementation (1%) of a control laboratory show containing adequate amounts of arginine for growth and reproduction increase thymic weight, cellularity, and thymic lymphocyte blastogenesis in rats and mice. In addition, arginine supplementation can alleviate the negative effect of trauma on these thymic parameters.

It has been demonstrated that arginine becomes an essential amino acid for survival and wound healing in arginine-deficient rats. This work showed that 1% arginine supplementation of non-deficient rats led to decreased weight loss on the first day post-injury, and increased wound healing in rats subjected to dorsal skin wounding.

L-arginine is a basic, genetically coded amino acid that is an essential amino acid for human development. It is a precursor of nitric oxide, and is synthesized by the body from ornithine. Arginine has been classified as a conditionally indispensable amino acid [140].

Arginine is used in certain conditions accompanied by hyperammonaemia. Neonatal Hyperammmonemia is a medical emergency requiring advanced planning, sophisticated facilities, and multidisciplinary teamwork. Urea cycle disorders (UCDs) are the primary cause of hyperammonemia during the vulnerable newborn period. Genetic defects in any of the first 5 enzymes of the pathway (carbamyl phosphate synthetase I (CPS), ornithine transcarbamylase (OTC), argininosuccinic acid lyase (ASL) argininosuccinate synthetase (ASS)), or a cofactor producer (N-acetyl glutamate synthase) result in accumulation of precursor metabolites including ammonia (figure 1). Because there is no effective secondary clearance system for ammonia, disruption of this pathway has a rapid clinical course. The catabolism normally present in the newborn period together with the immaturity of the liver combine to accentuate defects in these enzymes. This rapid accumulation of ammonia and other precursor metabolites results in acute cerebral edema with severe neurologic compromise [1-3], thus fast and effective treatment is key to improving the patient's outcome.

A clear, concise protocol is require to treat neonates with severe hyperammonemia caused by UCDs. In reviewing the experience of a number of clinicians who have cared for these patients, several stages of treatment become apparent. These include (1) recognition and supportive treatment, (2) bulk ammonia removal and pharmacological scavenging, (3) stabilization and catabolic reversal, and (4) transition to home management. These steps are undertaken to accomplish specific therapeutic goals and include rapidly clearing ammonia from the neonate's bloodstream, blocking the production of additional ammonia, removing excess nitrogen, and protecting the neurologic integrity of the baby. All of these goals should be pursued with thoughtful expediency in the context of the patient's clinical situation.

Arginine is regarded as intermediate component of the urea cycle. L-arginine or its salt (monohydrochloride) is used in alternative pathway therapy and it remains a mainstay of both acute and long-term treatment of inborn errors of urea synthesis.

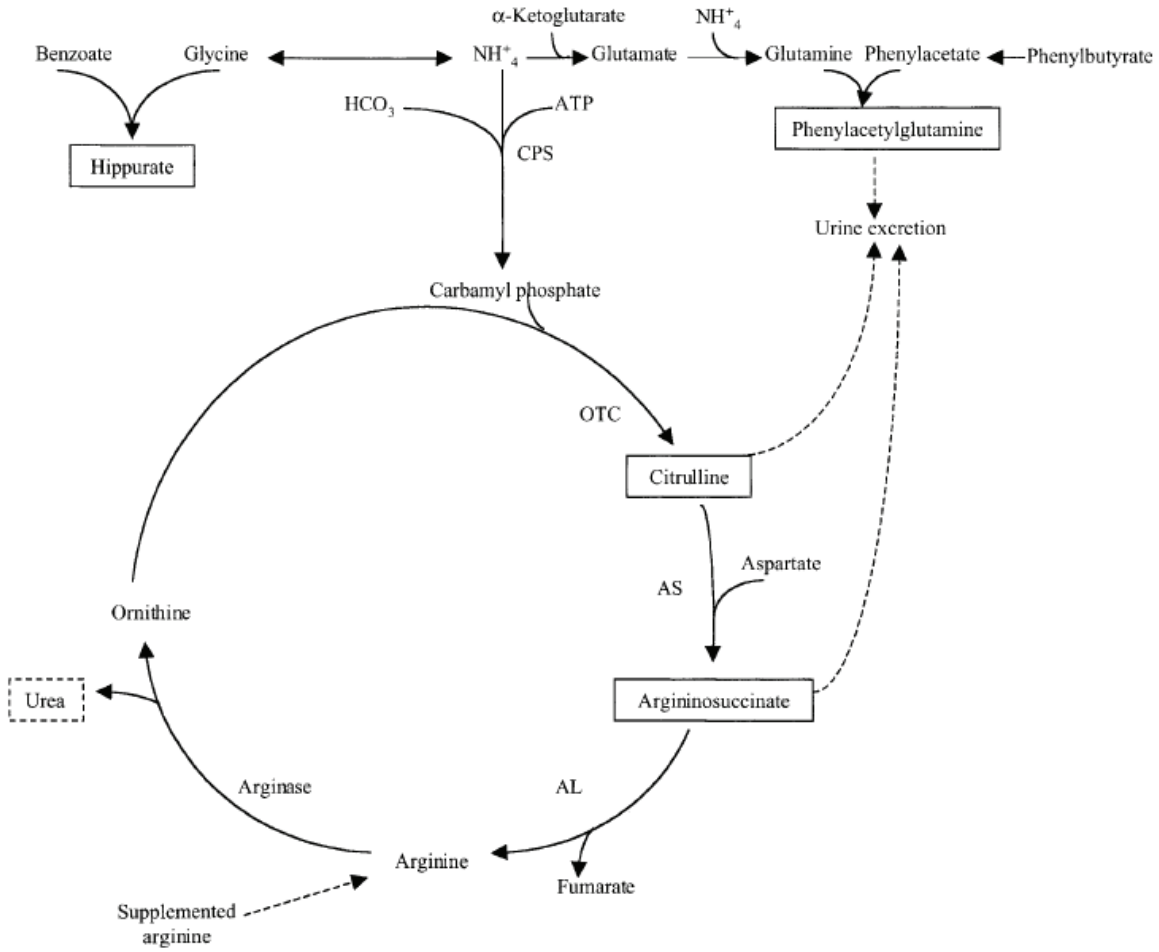

Figure 1: Urea cycle and intermediate components [10].

OTC= Ornithine Transcarbamylase

CPS= Carbamyl Phosphate Synthetase

AS= Argininosuccinate Synthetase

AL= Argininosuccinate Lyase

Evidence supports continued use at the currently recommended regimen (tables 1, 2). In the long-term treatment of inborn errors of urea synthesis, L-arginine free base is used to treat patients with ASS and ASL deficiencies at a dose of 3 to 4 mmol/kg/d (500 to 700 mg/kg/d) [4]. This dose has been well tolerated and is associated with plasma arginine levels 1.5 fold normal (mean 128 µmol/L). It also leads to further increases in plasma levels of citrulline (mean 3936 µmol/L), respectively, which are already markedly elevated in these disorders. There is significant excretion of citrulline and ASA in urine, representing 33 to 37% of waste nitrogen excretion in ASS deficiency and 52 to 59% in ASL deficiency. In ASL deficiency arginine therapy combined with protein restriction has proven very effective for long-term control.

Table 1: Use of alternative pathway therapy during intermittent hyperammonemic crisis in patients with urea cycle disorders [6].

Disorder	Drug administration	Sodium benzoate	Sodium phenylacetate	10% Arginine HCL
CPS or OTC deficiency	Priming infusion	0.250 g/kg or 5.5 g/m^2	0.250 g/kg or 5.5 g/m^2	0.20 g/kg (2 mL/kg) or 4.0 g/m^2
	Sustaining infusion	0.250 g/kg/24h or 5.5 g/m^2/24h	0.250 g/kg/24h or 5.5 g/m^2/24h	0.20 g/kg (2 mL/kg)/24h or 4.0 g/m^2/24h
Argininosuccinic acid synthetase deficiency	Priming infusion	0.250 g/kg or 5.5 g/m^2	0.250 g/kg or 5.5 g/m^2	0.60 g/kg (6 mL/kg) or 12.0 g/m^2
	Sustaining infusion	0.250 g/kg/24h or 5.5 g/m^2/24h	0.250 g/kg/24h or 5.5 g/m^2/24h	0.60 g/kg (6 mL/kg)/24h or 12.0 g/m^2/24h
Argininosuccinic acid lyase deficiency	Priming infusion	-	-	0.60 g/kg (6 mL/kg) or 12.0 g/m^2
	Sustaining infusion	-	-	0.60 g/kg (6 mL/kg)/24h or 12.0 g/m^2/24h
Arginase deficiency	Priming infusion	0.250 g/kg or 5.5 g/m^2	0.250 g/kg or 5.5 g/m^2	-
	Sustaining infusion	0.250 g/kg/24h or 5.5 g/m^2/24h	0.250 g/kg/24h or 5.5 g/m^2/24h	-

Table 2 summarizes the current recommendations for long-term alternative pathway therapy in the different urea cycle disorders. The basic principles are to give adequate nitrogen intake for growth, to provide arginine (or citrulline) as a semi-essential amino acid (except in arginase deficiency), and to stimulate alternative pathways of waste nitrogen excretion with phenylbutyrate (400-600 mg/kg/d) and arginine (400-700 mg/kg/d, in ASS and ASL deficiencies) [6].

Table 2: Long-term treatment of urea cycle disorders [6].

Disorder*	Citrulline	Arginine free base	Sodium phenylbutyrate†
CPS or OTC deficiency	0.170 g/kg/d or 3.8 g/m^2/d	-	0.450-0.600 g/kg/d if <20 kg; 9.9-13.0 g/m^2/d in larger patients
ASS deficiency	-	0.400-0.700 g/kg/d or 8.8-15.4 g/m^2/d	0.450-0.600 g/kg/d if <20 kg; 9.9-13.0 g/m^2/d in larger patients
ASL deficiency	-	0.400-0.700 g/kg/d or 8.8-15.4 g/m^2/d	May not be required
Arginase deficiency	-	-	0.450-0.600 g/kg/d if <20 kg; 9.9-13.0 g/m^2/d in larger patients
NAGS deficiency‡	0.170 g/kg/d or 3.8 g/m^2/d	0.170 g/kg/d or 3.8 g/m^2/d	0.450-0.600 g/kg/d if <20 kg; 9.9-13.0 g/m^2/d in larger patients

*Caloric requirement may be completed with a protein-free formula. In general, the minimum daily protein intake for growth was used: for 1 to 4 months, 1.6 to 1.9 g/kg/d; for 4 to 12 months, 1.7 g/kg/d; for 1 to 3 years, 1.4 g/kg/d. Daily protein intake may include an essential amino acid formula.

†If intolerant of phenylbutyrate, sodium benzoate and sodium phenylacetate can be given orally at a dose of 0.250 to 0.500 g/kg/d each.

‡N-carbamylglutamate may also be given at a dose of 0.320 to 0.650 g/kg/d.

CPS, NAGS, and OTC defects prevent the formation of citrulline from ornithine and carbamyl phosphate. This in turn decreases the synthesis of arginine, resulting in it becoming an essential amino acid. A block in ASS prevents the condensation of aspartate with citrulline, which accounts for 50% of the nitrogen incorporated into the pathway.

ASL deficiency blocks conversion of argininosuccinate to arginine. Therefore, arginine is also an essential amino acid in ASS and ASL deficiencies [4, 7]. Even in ASS and ASL deficiency, where there is a partially intact urea cycle, the body rapidly depletes its pool of urea cycle intermediates into which it normally incorporates nitrogen. Therefore, arginine serves as a therapeutic agent in UCDs. In CPS, NAGS, OTC, ASS, and ASL deficiency, arginine is used to restore its blood levels and prevent the breakdown of endogenous protein. In ASS and ASL deficiency it is used in larger amounts to “prime” the cycle to produce citrulline or argininosuccinate [8, 9]. This has the advantage of incorporating a substantial amount of nitrogen in compounds having a lower toxicity and higher renal excretion. In ASS (citrullinemia), 1 mole of nitrogen can be removed for every mole of arginine metabolized through the cycle, and this doubles in ASL to 2 mole.

Arginine is also a powerful immune stimulant agent. At one time, this was thought to be exclusively due to its growth hormone releasing properties, but arginine has been found to be a powerful immune stimulant and wound healing agent even in the absence of significant growth hormone release. Long-term oral administration of L-arginine reduces intimal thickening and enhances neoendothelium-dependent acetylcholine-induced relaxation after arterial injury. In addition, oral L-arginine improves interstitial cystitis symptom score. Arginine chloride has also been used as acidifying agent, where in severe metabolic alkalosis, intravenous doses (in gram quantities) have been calculated by multiplying the desired decrease in plasma-bicarbonate concentration (mEq per liter) by the patient’s body-weight (in kg) and then dividing by 9.6. In overdose, a suggested dose is 10 g intravenously over 30 minutes [16].

Arginine has also been used as various salt forms, such as the acetylasparaginate, aspartate, citrate, glutamate, oxoglutarate, tidiacicate, hydrochloride and timonacicate salts.

2. Transduction mechanisms and types of taste

All taste pathways are proposed to converge on common elements (center of figure 2) that mediate a rise in intracellular ([Ca^{2+}] in) followed by neurotransmitter (NT) release. **(a)** Sodium salts depolarize taste cells directly via Na^+ influx through amiloride-sensitive ENaC (dark blue). **(b)** Acids, in the form of protons (H^+), also permeate ENaC, activate H^+-activated cation (X+) channels (MDEG1 and, perhaps, ASIC [pale blue]) and inhibit apical K^+ channels (light green). **(c)** L-glutamate (L-Glu), which elicits umami taste, activates the taste form of mGluR4 (tmGluR4; red) (see figure 2), a GPCR that decreases cAMP levels via PDE activation. The decrease in cAMP may disinhibit cNMP-inhibited channels to elevate [Ca^{2+}] in. Other amino acids, such as arginine (L-Arg), activate ionotropic glutamate receptors (black), causing TRC depolarization. **(d)** Artificial sweeteners activate both ionotropic receptors (cyan) linked to cation channels, and GPCRs (magenta) linked via PLC to IP3 production and release of Ca^{2+} from intracellular stores. Natural sugars apparently activate GPCRs (orange) linked via AC to cAMP production which, in turn, may inhibit basolateral K^+ channels through phosphorylation by cAMP-activated protein kinase A (PKA). **(e)** Bitter compounds, such as denatonium and PROP, activate particular T2R/TRB isoforms (dark green), which activate gustducin heterotrimers. Activated a-gustducin stimulates PDE to hydrolyze cAMP, whereas $\beta\gamma$ subunits (e.g. $\beta 3$ $\gamma 13$) released from activated α-gustducin activate PLC$\beta 2$ to generate IP3, which leads to release of Ca^{2+} from internal stores. Other bitter compounds, including quinine and divalent cations, have been demonstrated to inhibit apical K^+ channels (yellow) in some species. AP, action potentials; DAG, diacylglycerol [96].

2.1 Bitter taste

Bitter stimuli, such as quinine, also act through G-protein-coupled receptors and second messengers. In this case, however, the second messengers cause the release of Ca^{2+} from the endoplasmic reticulum. The resulting build-up of calcium in the cell leads to depolarization and neurotransmitter release [97].

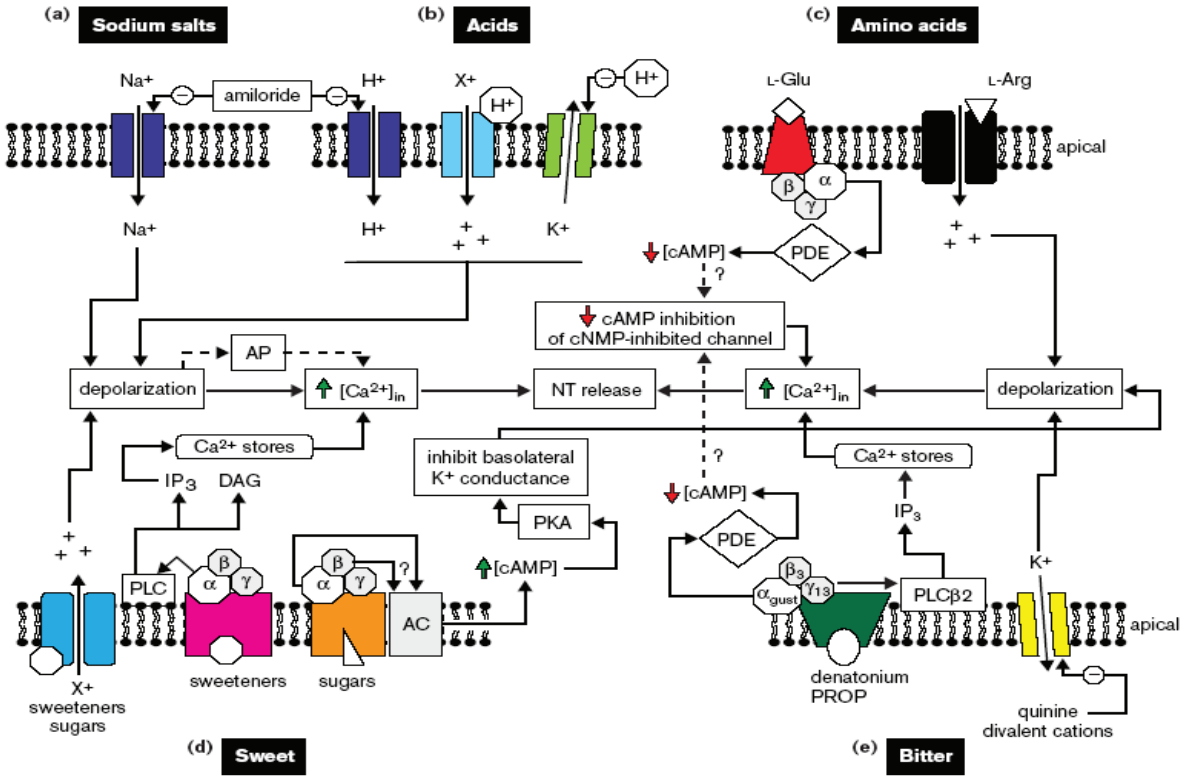

Figure 2: Proposed transduction mechanisms in vertebrate taste receptor cells [96].

2.2 Sweet taste

Sweet stimuli, such as sugar or artificial sweeteners, do not enter taste cells but trigger changes within the cells. They bind to receptors on a taste cells surface that are coupled to molecules named G-proteins. This prompts the subunits (α, β and γ) of the G-proteins to split into α and $\beta\gamma$, which activate a nearby enzyme. The enzyme then converts a precursor within the cell into second messengers that close K^+ channels indirectly [97].

2.3 Salt taste

Salts, such as sodium chloride (NaCl), trigger taste cells when sodium ions (Na^+) enter through ion channels on microvilli at the cells apical surface. (Sodium ions can also enter via channels on the cell's basolateral surface. The accumulation of sodium ions causes an electrochemical change called depolarization that results in calcium ions (Ca^{2+}) entering the cell. The calcium, in turn, prompts the cell to release chemical signals called neurotransmitters from packets known as vesicles.

Nerve cells, or neurons, receive the message and convey a signal to the brain. Taste cells repolarize, or "reset" themselves in part by opening K^+ channels so that potassium ions can exit [98, 99].

2.4 Acid taste

Acids taste sour because they generate hydrogen ions (H^+) in solution. Those ions act on a taste cell in three ways: by directly entering the cell; by blocking K^+ channels on the microvilli; and by binding to and opening channels on the microvilli that allow other positive ions to enter the cell. The resulting accumulation of positive charges depolarizes the cell and leads to neurotransmitter release [97].

2.5 Umami taste

Umami is a fifth basic taste quality primarily stimulated by L-glutamate, typically in the form of monosodium glutamate in the diet [100]. Amino acids such as glutamate are known to bind to G-protein-coupled receptors and to activate second messengers. But the intermediate steps between the second messengers and the release of packets of neurotransmitters are unknown [97]. In contrast to acids, which operate through a number of transduction pathways, the response to L-glutamate is probably much more limited in its mechanism of action.

2.6 Fat taste

Sense of taste informs the body about the quality of ingested foods. Five sub-modalities allowing the perception of sweet, salty, sour, bitter, and umami stimuli are classically depicted. The inborn attraction of mammals for fatty foods raises the possibility of an additional orosensory modality devoted to fat perception. For a long time, dietary lipids were thought to be detected only by trigeminal (texture perception), retronasal olfactory, and post-ingestive cues. Gustation also plays a significant role in dietary lipid perception [101]. Rats and mice exhibit a spontaneous attraction for lipids. Such behaviour raises the possibility that an orosensory system is responsible for the detection of dietary lipids.

2.7 Water

The sense of taste plays critical roles in nutrient identification and toxin avoidance. The ability to respond to hypoosmotic stimuli in mammalian taste receptor cells may reflect the importance of osmotic sensing by the gustatory system. Transduction for hypoosmotic stimuli involves water influx through aquaporins followed by activation of volume-regulated anion channels. The ability of these transduction elements to be regulated by natriferic hormones at the mRNA and protein level in other transporting epithelia suggest that the gustatory system may respond to extrinsic signals related to the restoration of salt and water balance. Plasticity in the peripheral gustatory system is consistent with the activity in the taste system being reflective of underlying nutritional status. Clearly, more research is needed to determine the link between nutrition, taste and the control of food and water intake [102].

3. Taste masking technologies

3.1 Taste masking with flavours, sweeteners, and amino acids

This technique is the foremost and the simplest approach for taste masking, especially in the case of paediatric formulations, chewable tablets, and liquid formulations. This approach is successful for many bitter and water soluble drugs. Artificial sweeteners and flavours are generally being used along with other taste-masking techniques to improve the efficiency of these techniques. Numerous pharmaceuticals such as dentifrices and mouthwashes applied to the oral cavity elicit unpleasant taste perceptions [118]. The unpleasant taste of certain formulations like mouthwashes and cough drops containing bitter tasting substances such as eucalyptus oil can be masked by adding fenchone, borneol, or isoborneol.

These taste masking agents significantly suppress the perception of unpleasant organoleptic sensations of the volatile oil [119]. The cooling effect of the taste masking agents also aids in reducing the bitterness. Sweetening compositions of di-D-fructofuranose 1, 2’2, 3’-di-anhydride are also useful for dentifrices, mouthwashes, and foods [120]. Menthol reduces the bitter taste.

Nonbitter dentifrices are prepared by sweetening benzethonium chloride with stevia-based sweetener extract and glycerin. It exhibits 100% bactericidal activity against E. coli [120]. Anethole and menthofuran in various dentifrices are not only used to mask the bitterness but also to improve the low temperature stability of the formulation. The use of some imitation flavours for masking the taste of ammonium chloride and other saline drugs has also been established. The various imitation flavour concentrates used are grape, maple, raspberry, and wild cherry, etc.. These have been compared to some of the official flavoured syrups and recognized as good masking agents for saline drugs [121].

The bitter taste of zinc acetate dihydrate in lozenge formulations can be masked by using saccharin, anethol-β-cyclodextrin complex and magnesium stearate followed by tableting with compressible polyethylene glycol and fructose [122]. Incorporation of anesthetizing agents such as sodium phenolate to an aspirin-medicated floss serves to numb the taste buds sufficiently for 4-5 seconds, rendering the bitter taste of aspirin imperceptible [123]. The combination of citric acid and sodium bicarbonate with certain flavours is used to mask the bitter taste of chlorpheniramine maleate and phenylpropanolamine HCl (orange flavour and cream flavour) [124], famotidine (lemon flavour) [125], and acetaminophen (cherry flavour) [126].

Alkali metal carbonates and bicarbonates in combination with mint flavour, aniseed flavour, and sweeteners are used to improve the taste of diclofenac. Glycyrrhizin and xanthan gum are used to improve the taste of extract containing pogostemi herba. Monosodium glycyrrhizinate together with flavours has been used to mask the bitter taste of guaifenesin [127]. Clove oil has been found to be a good taste-masking component to mask the bitter taste of a number of medicinals, particularly analgesics, expectorants, antitussives, decongestants, or their combination because of its spicy and slight anesthetic effect. To support the taste masking capabilities of clove, honey vanilla or artificial vanilla flavour is preferred.

Calcium carbonate, citric acid, or sodium bicarbonate may be included in the formulation if effervescence is required. Drugs, which can be taste masked by this composition, include acetaminophen, aspirin, ketoprofen, H2-blockers, etc..

A composition comprising of anethole, eucalyptol (provides cooling, by vaporization) and methyl salicylate (inhibits bitterness) can be used to mask the unpleasant taste of thymol, leaving the consumer with a pleasant taste perception [128]. Sodium citrate dihydrate, sodium saccharin, refined sugar, and flavours have been used to mask the bitter taste of ibuprofen when formulated as syrup with pyridoxine HCl [129]. Liposome-associated flavorents have been reported to mask the bitter taste of pharmaceuticals in aqueous suspensions.

Aspartame is used as a prominent sweetener in providing bitterness reduction. A very small concentration (0.8%) is effective in reducing the bitterness of 25% acetaminophen. Starch, lactose and mannitol have also exhibited taste-masking properties of caffeine [130]. Artificial sweeteners such as neohesperidine dihydrochalcone and hesperidine dihydrochalcone 4'-β-D glucoside have the ability to mask bitterness and saltiness by virtue of their lingering sweetness. A lingering sweetness provides taste masking, primarily because the taste profile of a bitter substance appears later in time than normal sugar sweetness generally lasts. Low levels of monoammonium glycyrrhizinate are reported to mask the bitter, harsh, and astringent taste in chewable multivitamins, cough/cold syrups, oral antibiotics, chewable analgesics and alcohol-based oral antiseptics. Several tasteless/sweetness inhibitors are being actively pursued as bitterness inhibitors. Lactisole, a sweetness inhibitor, possesses great potential in the taste masking of pharmaceuticals [131].

Anticholesterolemic saponin-containing foods, beverages, and pharmaceuticals are supplemented with amino acids (such as glycine and alanine) and flavors for bitterness control [132]. Protein like compositions, useful for improvement of liver disorders, severe burns, trauma, etc., having branched amino acid-modified proteins, are tasteless and odorless. Vitamin B oral solutions containing sugars, amino acids, and apple flavor are free from bitterness. Oral liquid compositions consisting of vitamin B, sod-5'-ribonucleoside (inosinate) and orange or fruit flavor also have improved taste. Oral liquid compositions containing theophylline salts are formulated with D-sorbitol, sodium saccharin, sodium glutamate, and vanilla essence to produce a solution that is less bitter than a theophylline solution [133].

3.2 Taste masking with lipophilic vehicles

3.2.1 Lipids

Oils, surfactants, polyalcohols, and lipids [48-51] effectively increase the viscosity in the mouth and coat the taste buds, and therefore they are potential taste masking agents. Guaifenesin has improved taste when mixed with carnauba wax and magnesium aluminium silicate and then melt-granulated [134]. Gabapentin has improved taste when coated with gelatin and then mixed with partially hydrogenated soybean oil and glyceryl monostearate [135].

3.2.2 Lecithin and lecithin-like substances

Formulations with a large excess of lecithin or lecithin-like substances are claimed to control bitter taste in pharmaceuticals [136]. Magnesium aluminum silicate with soybean lecithin is used to mask the unpleasant taste of talampicillin HCl. The drug is dissolved in or dispersed into an organic solvent such as chloroform. Lecithin is added to the solution or dispersion of the drug with stirring to give a blend. The blend is mixed with powdery excipients (e.g., magnesium aluminate metasilicate, synthetic aluminum silicate, lactose, mannitol, etc.), dried and granulated to give a taste-masked composition. Homogenated suspensions of phosphatidic acid and β-lacto-globulin from soybeans and milk, respectively, completely suppress bitter stimulants such as quinine, L-leucine, iso-leucine, caffeine, and papaverine HCl. More importantly, the suspension does not suppress sweet, sour, or salty taste [136].

3.3 Taste masking by physical barriers

This is the simplest and most feasible option to achieve taste masking. The coating acts as a physical barrier to the drug particles, thereby minimizing interaction between the drug and taste buds. For example, coating of pellets provides excellent taste masking while still providing acceptable bioavailability [49, 50]. Microemulsion technology has been used for taste masking of powders, chewable tablets, and liquid suspensions. Taste masking of ibuprofen has been successfully achieved by using the air-suspension coating technique to form microcapsules, which comprise a pharmaceutical core of crystalline ibuprofen and a methacrylic acid copolymer (Eudragit) coating that provides chewable taste-masked characteristics [138].

3.3.1 Carbohydrates

The taste of orally administered drugs can be masked by coating the drug with carbohydrates. Bitter solid drugs such as pinaverium bromide, a spasmolytic substance, has no bitter taste when formulated in an organoleptically acceptable manner by polymer coating with a mixture of cellulose or shellac and a second film forming polymer soluble at pH less than 5 [137]. A preparation of the antiulcerative drug propantheline bromide is coated on low substituted spherical hydroxypropylmethyl cellulose and further coated with ethyl cellulose to mask the unpleasant taste while readily releasing the active ingredients [137].

3.3.2 Zeolites

Bactericidal feeds for domestic animals generally impart bitter taste to the formulation and may create feeding aversion among the animals during the treatment. To improve the taste of such formulations, the active agent (tiamulin fumarate) may be dissolved in methanol, supported on mordenite-type zeolite or starch, dried, and further premixed with the supports to produce sustained-release, bitterness-free granules. The resulting formulation has stronger bactericidal effect on Mycoplasma, Staphylococcus and Corynebacterium [139].

3.4 Taste masking by inclusion complexation

In inclusion complex formation, the drug molecule fits into the cavity of a complexing agent, i.e., the host molecule, forming a stable complex. The complexing agent is capable of masking the bitter taste of drug by either decreasing its oral solubility on ingestion or decreasing the amount of drug particles exposed to taste buds, thereby reducing the perception of bitter taste. This method is most suitable only for low dose drugs. Van der Waals forces are mainly involved in inclusion complexes [139]. β-cyclodextrin is the most widely used complexing agent for inclusion type complexes. It is a cyclic oligosaccharide of minor sweetness obtained from starch.

3.5 Taste Masking by ion-exchange resins

Ion-exchange resins are high molecular weight polymers with cationic and anionic functional groups. The most frequently employed polymeric network is a copolymer of styrene and divinylbenzene. Ion-exchange resins are used in drug formulations to stabilize the sensitive components, sustain the release of the drug, enable disintegration tablets, and mask taste. Drugs are attached to the oppositely charged resin substrate, forming insoluble adsorbates or resinates through weak ionic bonding so that dissociation of the drug-resin complex does not occur under the salivary pH conditions. This suitably masks the unpleasant taste and odor of drugs. Drug release from the resin depends on the properties of the resin and the ionic environment within the gastrointestinal tract (GIT). Drug molecules attached to the resin are released by exchanging with appropriately charged ions in the GIT, followed by diffusion of free drug molecule out of the resins [117].

4. Taste masking assessment

4.1 In vivo approaches for taste assessment

Invivo studies, stimuli are applied on the tongues of either humans or animals. The stimulus interacts with receptors embedded in the membrane of the taste buds and the information is ultimately transduced as an electrical signal, which is further transmitted along the nerve fiber to the brain, where taste is perceived. Such studies include human taste panel studies, electrophysiological methods and animal preference tests.

4.1.1 Human taste panel studies

Human taste panel studies evaluate tastants (food, chemicals, drugs and so on) by estimating the gustatory sensation responses in healthy human volunteers within well-controlled procedures. Such studies are therefore also known as physiological evaluation, psychophysical evaluation, gustatory sensation tests, sensory tests or taste trials. They are sensitive measures of taste and are statistically designed to minimize bias and variable responses within and between human volunteers.

Well-established methodologies for performing sensory analysis can be broadly divided into five types, namely discrimination tests, scaling tests, expert tasters, affective tests and descriptive methods. The methods are excellently discussed elsewhere [103]. Volunteers assess the taste quality and intensity of standard and test stimuli on different adjective scales.

Such scales include various properties of the sample, such as overall intensity, sweet, sour, salty, bitter, metallic, cooling, hot, spicy, burning, anesthetic, astringent, medicinal, minty/menthol, warming, sharp, alcohol, painful, irritating, stinging, dry, peppery and paper [104]. Each adjective can be rated on an intensity scale ranging from zero (none at all) to four or perhaps even up to nine points (with the highest point on the scale referring to the maximum intensity for each parameter) on provided score sheets. To develop temporal profiles, the intensity of adjectives is determined at different time points.

The perception of taste of medicines has been shown to be different between adults and children and will probably differ between healthy and sick children. Thus, ideally taste should be assessed in children, but there may be some ethical concerns to perform taste studies in healthy children unless the study is a ‘swill and spit’ one with drugs known to have a good safety profile. The EU ad hoc committee considering ethical aspects of clinical trials in children has stated: ‘In principle, healthy children should not be enrolled as healthy volunteers, because they cannot consent and are vulnerable like children with a disease or condition.

Studies should not be performed in children when they can be performed in adults. Exceptions could be where healthy children participate in palatability testing such as swill and spit taste testing for a new flavoured medicine. For many drugs, e.g. cytotoxics, it would be considered unethical to enroll healthy volunteers, even in ‘swill and spit’ tests. These should have taste assessed when administered to children with the illness to be treated and the study should preferably be embedded within another clinical study [142].

4.1.2 Animal preference tests

Bottle preference and conditioned taste aversion tests are used for determining taste preference and concentration-response properties of tastants by animals. Rats, mice, cats and dogs can be used for conducting such preference determination tests.
Attempts have been made to develop methodologies that can produce robust behavioural tests, capable of providing data comparable with those obtained from physiological investigations. A brief contact procedure has been studied to evaluate the ability of rats to detect the presence of a weak bitter compound dissolved in a strong sucrose solution. These results demonstrate the acute ability of rats to discriminate by taste, not only the presence but also the concentration of a dilute bitter compound dissolved in a sucrose solution [105].

4.1.3 Electrophysiological methods

Electrophysiological recordings from animals, primate and human taste nerves have provided insights into the physiology of taste sensation. Responses of tastants from single glossopharangeal or chorda tympani nerve fibers or nerve bundles can be utilized for taste assessment [106]. Mice, bull frogs (Rana ctesbeiana) or gerbils (Meriones unguiculatus) [104] have all been described in the literature in this respect.

In these tests, the animal is anaesthetized; following which electrodes are implanted in the chorda tympani nerve bundle and/or glossopharyngeal nerve. Tastant solutions are then passed over the tongue for a controlled period. Electrophysiological recordings from the chorda tympani and/or glossopharyngeal nerve provide a means of directly measuring the temporal profiles or dose response curves of taste stimuli [107].

4.2 In vitro approaches for taste assessment

Release studies are commonly used in taste assessment to measure the effectiveness of coating and complexation within a formulation. They are indirect methods for assessing taste because the methods do not contribute to the evaluation of taste and sweetness of the drug product.

Novel drug release apparatus and pharmacopoeial apparatus have both been adapted to simulate buccal dissolution of dosage forms so as to compare taste in different pharmaceutical formulations. Such novel apparatus and methods for drug dissolution or release studies tend to simulate the release of bitter or undesirable tasting drug in the mouth [108]. The in vitro biochemical assay of gustducin and/or transducin can also be used for the high-throughput taste assessment of new molecular entities [109].

4.2.1 In vitro drug release studies

Pharmacopoeial release tests have been modified by altering the chemical composition of the dissolution media (e.g. artificial saliva) and reducing the size of the basket screen size (screen size < 0.381 mm square opening) to prevent particles from escaping [108]. Taste masking is achieved when, in the early time points from 0 to 5 min, the drug substance in the dissolution medium is either not detected or the detected amount is below the threshold for identifying its taste.

Drugs can be analyzed either spectrophotometrically or using HPLC. Of these, HPLC is generally preferred, especially when testing is performed in the presence of UV-absorbing components, such as flavourings and sweeteners. Furthermore, the drug signal is frequently indistinguishable from background in the UV estimation when there is a high excipient-drug ratio in the taste-masked formulations.

The degree of masking of bitter taste from fine granules has been evaluated using a simplified in vitro dissolution test [108, 109]. Either fine granules, dry syrup or drug powder are gently mixed with a small amount of distilled water in a syringe by revolving the syringe. Thereafter, concentrations of drug substance in the ultrafiltrate, obtained by passing through a pore size of 0.45 mm, are determined either spectrophotometrically or using HPLC.

4.2.2 In vitro assay methods

Gustducin and transducin are guanine nucleotide-binding regulatory proteins expressed in taste receptor cells (TRCs). Gustducin is selectively expressed in 20-30% of TRCs in the palate and all taste papillae, and in apparent chemosensory cells in the gut and the vomeronasal organ.

Most bitter stimuli can activate both transducin and gustducin, and this activation depends upon receptors in the taste-bud membrane. The activation of gustducin and/or transducin in the presence of the taste-budmembrane can be measured to identify certain bitter tastants, determine molecular mode of action, quantitatively determine potency profiles and screen chemical libraries for potential bitterness inhibitors [110]. Not all the bitter compounds demonstrate in vitro activity (gustducin independent taste modifiers, e.g. caffeine and aristolochic acid), which could be due to the presence of multiple transduction pathways.

Furthermore, gustducin and/or transducin are not activated in the presence of sucrose, glycine, monosodium glutamate, citric acid or potassium chloride.

4.3 Biomimetic taste sensing systems (BMTSSs)

The use of multivariate data analysis (MVDA) combined with sensors that have partially overlapping selectivities, has been demonstrated to be a powerful tool in taste measurement technology. Such systems, often referred to as artificial senses, emulate biological taste reception at the receptor level, the circuit level and the perceptual level (figure 3). BMTSSs have been marketed as taste sensors, or electronic tongues or e-tongues [112]. These instruments employ electrochemical sensors coupled with chemometric methodologies to perform qualitative and quantitative analyses of organoleptic and chemical properties of substances and products.

The data can be processed using MVDA, either to search for correlation within the data or to develop predictive models. BMTSSs have been shown to be globally selective for detecting and quantifying specific classes of chemical compounds [113]. They do not discriminate minute differences in the structure of compounds but can transform molecular information from interactions with biological membranes into several types of group, that is, taste intensities and qualities. Taste sensors therefore act as tools to reproduce the complex and comprehensive taste sense of humans.

Global selectivity signifies the quantification of a combination or mixture of various types of substances that result in a compound effect, such as a synergistic effect or suppression effect amongst the substances. Flavour, odour and contamination by organic substances (such as humic substances) are some examples of this compound effect.

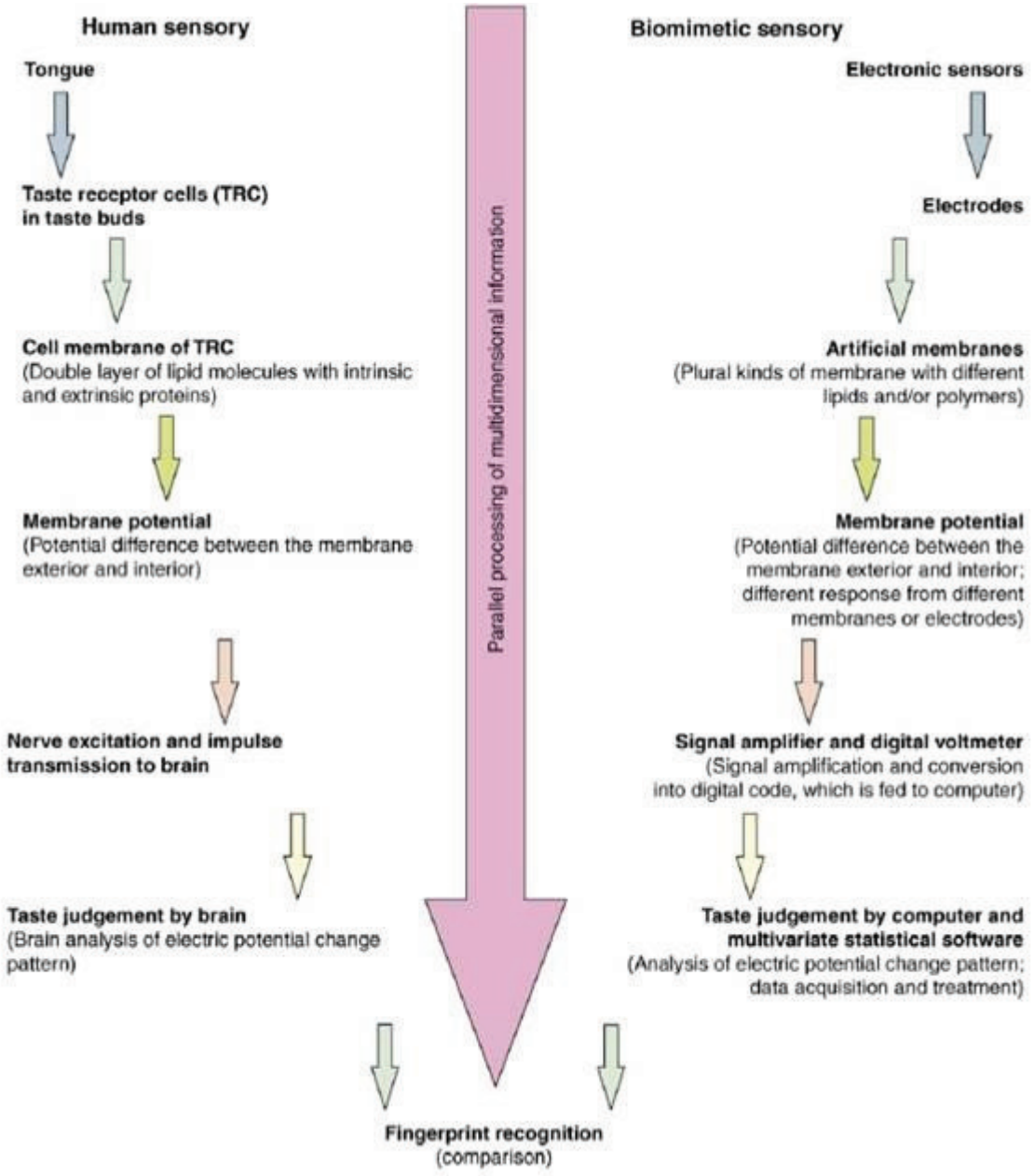

Figure 3: Development of taste sensors on the basis of mechanisms found in biological systems [141].

Furthermore, taste sensors have the ability of molecular recognition to distinguish chemical substances even in the same group of taste; for example, the patterns for hydrochloric acid, citric acid and acetic acid are slightly different [113]. Thus, the global selectivity concept is based on the recognition of the response patterns that characterize different classes of chemical compounds through the use of electronic sensor(s).

4.3.1 Lipid membrane taste sensors (LMTSs)

LMTSs capitalise upon the properties of lipids, which participate in the natural process of taste. The sensors are formed by dispersing the lipid compound responsible for transducing the signal on to a polymeric matrix that is normally non-conducting, such as polyvinyl chloride. Such sensors analyze, in a non-specific manner, detected signals and hence can extract the inherent taste characteristics of substances [112].

4.3.2 The taste-sensing systems SA401 and SA402

Two specific systems SA401 and SA402 have been developed by Anritsu Corporation together with researchers at Kyushu University in Japan. The detecting sensor part of the systems consists of seven (SA401; Anritsu Co., Ltd, Japan; figure 4a) or eight (SA402; Intelligent Sensor Technology, Inc., Japan; (figure 4b) electrodes made of lipid–polymer membranes.

Different types of lipid are used for preparing the membrane (e.g. oleic acid, oleyl amine, decyl alcohol and so on) depending upon the material being measured. Each lipid is mixed in a test tube containing polyvinyl chloride and dioctyl phenyl phosphonate as a plasticizer, dissolved in tetrahydrofuran, and dried on a glass plate at 30°C to form a transparent thin film, almost 200 μm thick. Lipid or polymer membranes are fitted on a multichannel electrode that acts as the detecting electrode.

4.3.3 Astree electronic tongue

The Astree electronic tongue system (Alpha M.O.S.) is a taste-sensing instrument equipped with a seven-sensor probe assembly (figure 3c) for qualitative and quantitative analysis [114]. It is fully automated, with 16 or 48 positions for formulation samples. The probes consist of a silicon transistor with proprietary organic coatings that govern the sensitivity and selectivity of the probe. Tastant molecules in the sample interact with the proprietary organic coating, which modifies the physical properties of the sensor, resulting in potential variations. The measurement is potentiometric, with readings taken against an Ag/AgCl reference electrode.

Each probe is cross-selective to enable coverage of the full taste profile. The system samples, quantifies, digitizes, records and processes potentiometer readings with multivariate statistical tools integrated with software.

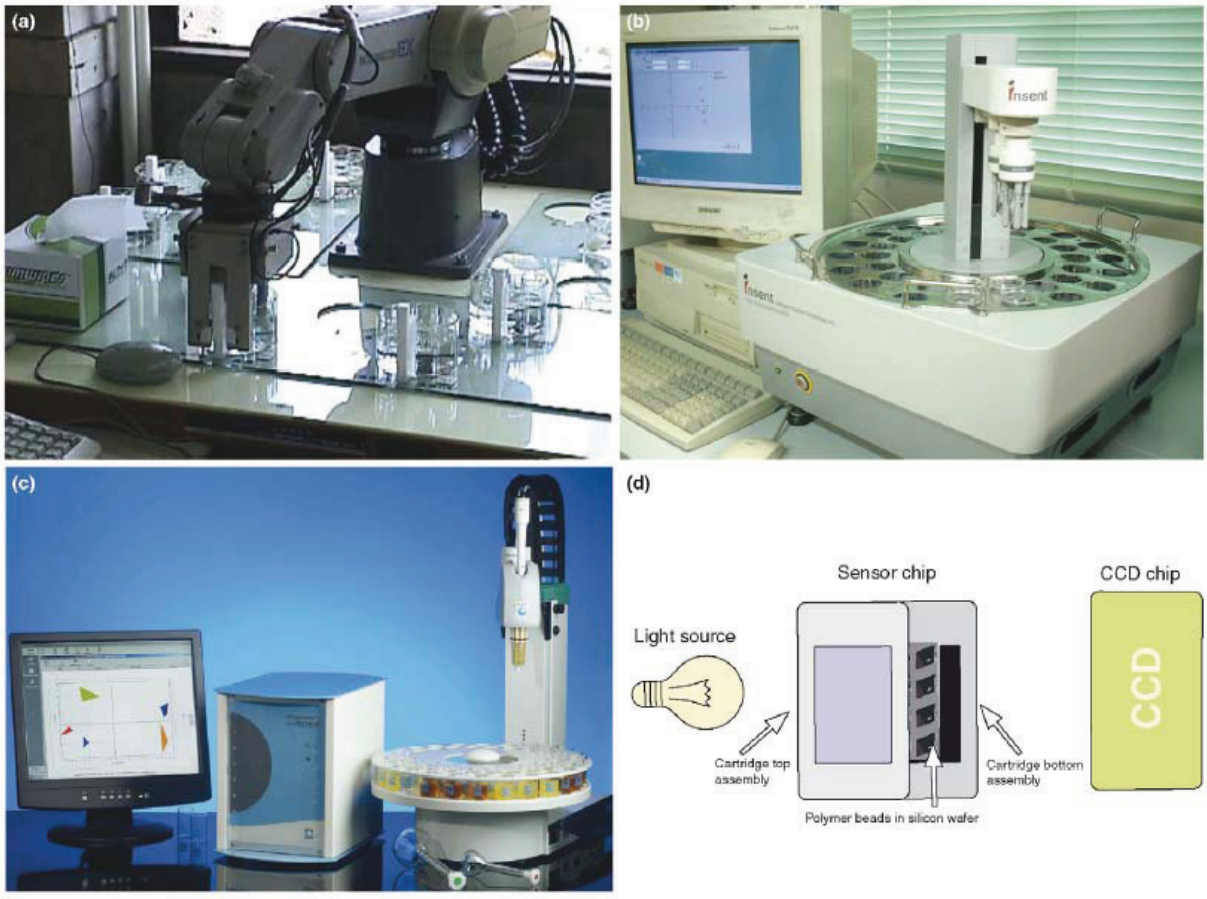

Figure 4: Commercially developed and developing taste sensors. (a) Taste sensor SA401 developed by Anritsu Corp. (b) Taste sensor SA402B developed by Intelligent Sensor Technology (Insent Corp). (c) Astree electronic tongue developed by Alpha M.O.S. (d) a schematic of the electronic tongue developed by the University of Texas and Vusion [141].

4.3.4 Ion-sensitive field effect transistors

FET taste sensors are prepared by pasting artificial lipid-polymer membranes of the same composition, as in LMTS above, on to the gate of a FET.

The FET taste sensor has the same sensitivity to taste substances as LMTS, but the potential reproducibility is less than that for LMTS and the lifetime is shorter for miniaturized devices [113]. Ion-sensitive FET sensors determine ions present in solution (e.g. mineral water and wines).

4.3.5 Voltammetric sensors

The voltammetric electronic tongue, developed by S-Sence, consists of four working metal electrodes made of gold, iridium, platinum and rhodium, an Ag/AgCl reference electrode and a stainless steel counter electrode [115]. A relay box enables the working electrodes to be connected consecutively, to form four standard three-electrode configurations. The potential pulses steps are applied by a potentiostat which is controlled by a personal computer (PC). The PC is used to set and control the pulses, measure and store current responses, and to operate the relay box. Voltage pulses are applied to the working electrode and the resulting current is measured.

4.3.6 Alternative electronic tongue

The electronic tongue initially developed by the University of Texas consists of a light source, a sensor array and a detector [116]. The light source shines onto chemically adapted polymer beads arranged on a small silicon wafer, which is known as a sensor chip. These beads change colour on the basis of the presence and quantity of specific chemicals. The change in colour is captured by a digital camera and the resulting signal converted into data using a video capture board and a computer (figure 3d). The technology can be applied to the measurement of a range of chemical compounds, from the simple, such as calcium carbonate in water (which effects water hardness), through to complex organic compounds, such as haemoglobin in blood and proteins in food. Moreover, it is helpful in discriminating mixtures of analytes, toxins and/or bacteria in medical, food/beverage and environmental solutions. As a result, the electronic tongue has many potential uses in the food, beverage, chemical and pharmaceutical industries. Vusion, Inc. is developing a chemical analyzer and sensor cartridge, based upon the electronic tongue technology of University of Texas, which can instantly analyze complex chemical solutions [116]. The analyzer consists of a customized housing into which the sensor cartridge can be placed and exposed to liquid chemicals within a process plant.

C Results and Discussion

1. L-arginine HCL 10% solution

1.1 Introduction

The major aim of the present studies has been the development of age appropriate dosage forms for children suffer from urea cycle disorders disease. The challenges are the taste masking of L-arginine, which has umami and bitter taste and its stability in liquid dosage forms. As also newborn infants are affected by the disease, one of the most important issues in the development of medicinal products for paediatric patients is the most appropriate dosage form in relation to age. The oral route of administration is commonly used for dosing medicinal products to paediatric patients and consequently many medicinal products should be available in both liquid and solid oral dosage forms. The variety of different oral dosage forms available, such as solutions, syrups, suspensions, powders, granules, effervescent tablets, orodispersible tablets, chewable tablets and gums, mini tablets, innovative granules, conventional immediate release, modified release tablets and capsules, make this route extremely useful for the administration of medicinal products to paediatric patients of a wide age range. Liquid formulations include solutions, syrups, suspensions and emulsions are most appropriate for younger paediatric patients (e.g. birth to 8 years) who are unable to swallow capsules or tablets. The dose volume is a major consideration for the acceptability of a liquid formulation. Typical target dose volumes for paediatric liquid formulations are < 5 ml for children under 5 years and < 10 ml for children of 5 years and older. However, the more palatable the formulation, the higher the dose volume which will be tolerated. Large volume doses may be inconvenient for both patient and carer. In the EMEA reflection paper “Formulations of choice for the paediatric population” [5], the suitable peroral dosage form for preterm newborn infants, term newborn infants and toddlers is solution/drops, as seen in table 3.

Furthermore, it is the standard route of administration in the long-term treatment of inborn errors of urea synthesis and the aim of long-term therapy has been to maintain metabolic control with plasma ammonia concentrations less than twice normal and plasma glutamine levels <1000 µmol/L.

Table 3: Matrix: Route of administration/dosage form vs. age; For the early ages the code indicates mainly the applicability of the route and the dosage form: 1 not applicable, 2 applicable with problems, 3 probably applicable, but not preferred, 4 good applicability, 5 best and preferred applicability. For the higher ages more or less all dosage forms might be principally applicable, but with increasing age the preference of the children becomes more important: 1 not accepted, 2 accepted under reserve, 3 acceptable, 4 preferred acceptability, 5 dosage form of choice [5].

Route Dosage Form	***Preterm newborn infants***	***Term newborn infants (0d-28d)***	***Infants and Toddlers (1m-2y)***	***Children (preschool) (2-5y)***	***Children (school) (6-11y)***	***Adolescents (12-16/18y)***
Peroral						
Solution/ Drops	2	4	5	5	4	4
Emulsion/ Suspension	2	3	4	5	4	4
Effervescent DF*	2	4	5	5	4	4
Powders/ Multiparticulates	1	2	2	4	4	5
Tablets	1	1	1	3	4	5
Capsules	1	1	1	2	4	5
Orodispersable DF	1	2	3	4	5	5
Chewable tablets	1	1	1	3	5	5
Nasal						
Solution	3	4	4	4	4	4
Semisolid DF	2	3	3	4	4	4
Rectal						
Suppositories	4	5	5	4	3	2
Rectal Enema	5	4	4	3	3	2
Rectal capsules	2	3	4	4	4	3
Topical/ transdermal						
Ointment, Cream, Gel	4	4	4	5	5	5
Liquid DF	4	4	4	5	4	4
Transdermal Patch	1	2	2	4	4	5
Parenteral						
i.v. Solution	5	4	4	4	4	3
i.m.	3	3	3	4	4	3
s.c.	4	4	4	4	4	3
Pump system	5	4	4	4	4	3
Pulmonary						
Nebuliser	2	3	4	5	4	3
MDI / Spacer	1	3	4	5	4	4
DPI	1	1	3	4	5	5
Ocular						
Eye drops	3	4	4	4	5	5
Semisolid DF	2	3	4	4	4	4

*DF: Dosage Forms

For the acute-term treatment of inborn errors of urea syntheses with L-arginine there are R-Gene® 10 (10% Arginine Hydrochloride Injection, USP), L-Arginin-hydrochlorid 21% Braun and L-Arginin-hydrochlorid 21.07% Fresenius Kabi available. To avoid intravenous irritation these drug should be well diluted. There is no set dilution, but the required volume of drug should be diluted to give a total volume of at least 20 times the drug volume. L-arginine HCL will be more than adequately diluted even at maximum dose.

The need for L-arginine HCL oral solution in the long-term treatment of inborn errors of urea syntheses results from the fact that there are no oral formulations for L-arginine HCL and during this phase preparations are made to facilitate continuing treatment of the patient in the home environment. Careful consideration should be given to the route of feeding. Arginine is medical supplement rather than drug approved by FDA or EMEA, and explanatory documentation will usually be necessary [10]. So far, in clinical practice the respective hospital or community pharmacies are the provider of the oral L-arginine HCL formulation.
As there is no recommended or fixed concentration for the strength of the oral L-arginine HCL formulation, contents may differ. The strength of L-arginine HCL solution was selected to be 10% depending on the oral dose given at the hospital and from the literature survey and it was (500 mg/kg/day).

1.2 Formulation development

When developing an oral liquid dosage formulation, consideration is first given to the characteristics of the active drug. The major challenges in developing oral liquid dosage forms are (i) the stability of a drug in solution, (ii) the solubility of a drug at the required level, and (iii) an acceptable taste. It is the effective use of excipients, which allows formulators overcome these challenges.

Additionally, an excipient’s compatibility with a drug substance in the solid state cannot infer the same compatibility in solution. However, if the mechanism of degradation of the drug is understood, the process of selecting which excipients to use in a solution will be much easier.

Finally, some knowledge of the drug's physical and chemical characteristics are essential in order to choose the proper excipients effectively. Ideally, the pH at which the drug is most stable would also be close enough to the solubility for delivering the desired dose in approximately 5 mL. Requiring patients to take more than 10 mL at a time may not be advisable because of lower patient compliance. Several aspects had to be taken into consideration for the development of a L-arginine HCL solution. The concentration should be suitable for correct dosing to avoid unnecessary doing errors through calculation mistakes.

In drug formulation development, preformulation strategy plays a vital role in order to obtain a proper and stable dosage form. This approach assists the formulator to reduce preparing unnecessary formulations leading to reduced cost and time effectiveness. In general, it reduces the number of experimental formulations while the effect of each factor to the stability of the formulation can still be achieved. These techniques have been used for several drugs such as phenol, pyridoxal hydrochloride and furosemide [11-14].

The full factorial block design is one of widely used methods for studying the effect of each variable during the drug development. Attempting to study several variables simultaneously results in the need to prepare a number of formulations. If n is the number of such variables, 2^n is the number of formulations required for a study [15]. In this study the full factorial block design was conducted which was similar to the 2^3 full factorial design [11]. In the first preformulation study, these factors included antioxidant, co-solvent (stabilizer agent) and chelating agent, because L-arginine in solution form is expected to undergo oxidization and degradation [24]. According to the block design, the effects of the presence and absence of each factor in the preparation were investigated.

Glycerin is used in a wide variety of pharmaceutical formulations including oral, otic, ophthalmic, topical, and parenteral preparations. In topical pharmaceutical formulations and cosmetics, glycerin is used primarily for its humectant and emollient properties. In parenteral formulations, glycerin is used mainly as a solvent or co-solvent.

In oral solutions, glycerin is used as a solvent, sweetening agent, antimicrobial preservative, stabilizing agent, and viscosity enhancing agent. It is also used as a plasticizer in film coatings. Glycerin is additionally used in topical formulations such as creams and emulsions. It is also employed as a therapeutic agent in a variety of clinical applications, for example as a mild laxative, and is also used a food additive. When used as an excipient or food additive, glycerin is not usually associated with any adverse effects, but sometimes despite osmotic effects, and therefore is generally regarded as a nontoxic and nonirritant material [23].

Sodium bisulfite is widely used as an antioxidant at intermediate pH in oral, topical and parental pharmaceutical formulations; it is also widely used in food products, given this, many investigators have studied the use of sodium bisulfite for stabilization of drugs, but sodium bisulfite and other sulfites have been associated with a number of anaphylactic reactions [23].

Other factor which assists L-arginine stability is EDTA. Edetic acid and edetates are primarily used as antioxidants synergistic, sequestering trace amounts of metal ions, particularly copper and iron that might otherwise catalyze autoxidation reactions. They may be used alone or in combination with antioxidants, the usual concentration employed being in the range 0.005-0.1% w/v.
They possess some antimicrobial activity but are most frequently used in combination with other antimicrobial preservatives owing to their synergistic effects. Disodium EDTA is used in a greater number and variety of pharmaceutical formulations than the free acid. It is poorly absorbed from the gastrointestinal tract and associated with few adverse effects when used as excipient in pharmaceutical formulation, generally regarded as an essentially non-toxic and non-irritant material [23]. Disodium EDTA is recommended to be added in L-arginine formulations.

Domiphenbromide, also known as N,N-Dimethyl-N-(2-phenoxyethyl)-1 dodecanaminium bromide, is a quaternary ammonium salt. It was found to be a suitable antimicrobial agent and it has antimicrobial activity through wide range of pH, especially at intermediate pH. 6.5 pH which is a neutral pH was selected for L-arginine HCL aqueous formulations.

This is expected to import L-aginine HCL better taste and stability in a aqueous form, for this reason used in oral L-arginine HCL solution [25].

1.3 Taste masking of L-arginine in solution

Paediatric patients are able to recognize sweetness from an early stage of life and are also able to recognize sweet taste in mixtures and estimate the strength or degree of sweetness. The same is true for saltiness. However, their ability to distinguish and recognize both tastes in a mixture depends on the age of the child but is limited compared to adults either due to immature analytical abilities or because the gustatory system processes mixtures differently to that of the adults. Children seem to prefer higher levels of sweetness than adults and there appear to be transient gender differences only in children with 4-12 year-old girls more sensitive to sweetness and saltiness than boys [5].
Factors, such as food selection of adults and peers can have strong effects on children's flavour preferences. Cultural influences can also have strong effects on children's attitudes and preferences toward even the basic tastes and flavours. Market research has revealed standard combinations of specific sweeteners with relevant flavours, which may vary by country and target market. National favourites include "bubble-gum" and "grape" in the United States, "citrus" and "red berries" in Europe and "liquorice" in Scandinavia. A bubble-gum or cherry flavour in combination with a high intensity sweetener may suit the US paediatric market, while a less intense sweetness may be more appropriate for Japan [5].

For this reason, cherry syrup, raspberry syrup and honey were selected as the most suitable flavouring syrups for umami and bitter taste masking of L-arginine as a paediatric medication, because they are mostly used in Europe for taste masking of bitter drugs for the paediatric population.
Honey is carbohydrate-rich syrup produced by bees, primarily from floral nectars. Fructose and glucose are the major components but a large number of other chemical compounds are present in small quantities. Moisture content and water activity are low. The British Pharmacopeia (1993) and European Pharmacopeia (2008) provide a monograph for purified honey that adequately defines the substance.

Honey has an extensive history of traditional human medicinal use, in a large number of societies. It may be used alone or in combination with other substances, and has been administered both orally and topically and is also used as an active medicinal compound. In modern pharmaceutical practice it is widely used as an excipient ingredient and is already used by the TGA for this purpose, in addition to, it is used in many oral pharmaceutical formulations to mask the bitterness of many active ingredients due to it is composition that contains many types of sugars and flavouring aids. In oral rehydration products honey is widely used, typically for oral preparations in infants and young children to mask the unpleasant taste.

Sucralose was used as intense sweetening agent in masking the taste of L-arginine, because high concentrations of intense sweeteners such as sodium saccharine or aspartame are sometimes used but may be unsuccessful in masking bitter taste in paediatric formulations. These sweetening agents appear to develop a bitter aftertaste at high concentrations. The intensity of sweetness and bitter taste masking capacity at relative low concentration levels may be enhanced by the addition of a sodium salt to the mixture, presumably by blocking bitterness and thereby releasing sweetness [5].

Sucralose is used as a sweetening agent in beverages, foods, and pharmaceutical applications. It has a sweetening power approximately 300-1000 times that of sucrose. It has no nutritional value and is non-cariogenic. It is obtained from natural cane or beet sugar by the chlorination of sucrose. Its glycosidic bond is resistant to acid and enzymatic hydrolysis; as a result, it is non-caloric and non-carcinogenic.

Sucralose is generally regarded as a nontoxic and nonirritant material and is approved, in a number of countries, for use in food and pharmaceutical products. Following oral consumption, sucralose is predominantly not absorbed and is excreted in the feces. The WHO has set an acceptable daily intake for sucralose of up to 15 mg/kg body-weight. In addition to, the sweetening effect of sucralose is not reduced by heating and food and pharmaceutical products containing sucralose may be subjected to high temperature processes such as pasteurization and sterilization [23]. The shelf-life of sucralose is very long, with no significant degradation over a long period of time, excellent stability across a wide pH range.

It is important to mention, as with the other high-intensity sweeteners, the sweetness intensity of sucralose decreases at higher concentration.

1.4 First preformulation study

The master formula of L-arginine solution consisted of 10% (w/v) L-arginine HCL, 0.01% (w/v) domiphenbromide as a preserving agent and purified water. In this study, 3 factors were chosen to be evaluated; sodium bisulfite as an antioxidant agent, disodium EDTA (Na_2EDTA) as a chelating agent and glycerin as a (co-solvent).

Thus, the total number of the experimental formulations was 8 (2^n), where n was the number of factors studied. The design was similar to that described by Lewis [11]. However, sodium bisulfite, disodium EDTA and glycerin were added (if presented) at concentrations of 1% (w/v), 0.1% (w/v) and 10% (w/v), respectively.

The plus or minus designations in the block referred to the presence or absence of the variable in the formula or referred to the higher or the lower of particular parameter. For example, apart from ingredients specified in the master formula, formulation F5 comprised sodium bisulfite and disodium EDTA, table 4.

Table 4: Full factorial block design showing factors studied after 35 days of storage at different storage temperatures (n=3).

		Sodium bisulfite (−)	Sodium bisulfite (+)
Glycerin −	Disodium EDTA −	F1	F2
	Disodium EDTA +	F4	F5
Glycerin +	Disodium EDTA −	F3	F6
	Disodium EDTA +	F7	F8

(+), presence; (−), absence

Table 5 summarizes the eight formulations prepared according to the design given from table 4. Three bottles of each formulation were prepared and kept in amber glass bottles (n=3), stored at either 5°C, 25°C, or 40°C for 35 days.

Table 5: Eight formulations of L-arginine HCL solution in first preformulation study in relevant to the full factorial block design (n = 3).

Component	F1 %w/v	F2 %w/v	F3 %w/v	F4 %w/v	F5 %w/v	F6 %w/v	F7 %w/v	F8 %w/v
L-arginine HCL	10	10	10	10	10	10	10	10
Domiphenbromide	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Sodium bisulfite	-	1	-	-	1	1	-	1
Disodium EDTA	-	-	-	0.1	0.1	-	0.1	0.1
Glycerin	-	-	10	-	-	10	10	10
Purified water (ml) to	100	100	100	100	100	100	100	100
pH adjusted to	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5

Prefomulation approach utilizing the full factorial block design was employed for the formulation development and stability testing of L-arginine HCL 10% solution. Factors expecting to affect the stability of L-arginine HCL were evaluated, i.e., stabilizer, antioxidant and chelating agent. Eight formulations were prepared according to the block design. The stability of the preparations was tested after 35 days of storage. The data of preformulation study will be used for formulation development. The formulations from F1 to F8 that stored at 5°C and 25°C were stable and within the standard range of L-arginine content (97-103%), as seen in figures 5 and 6.

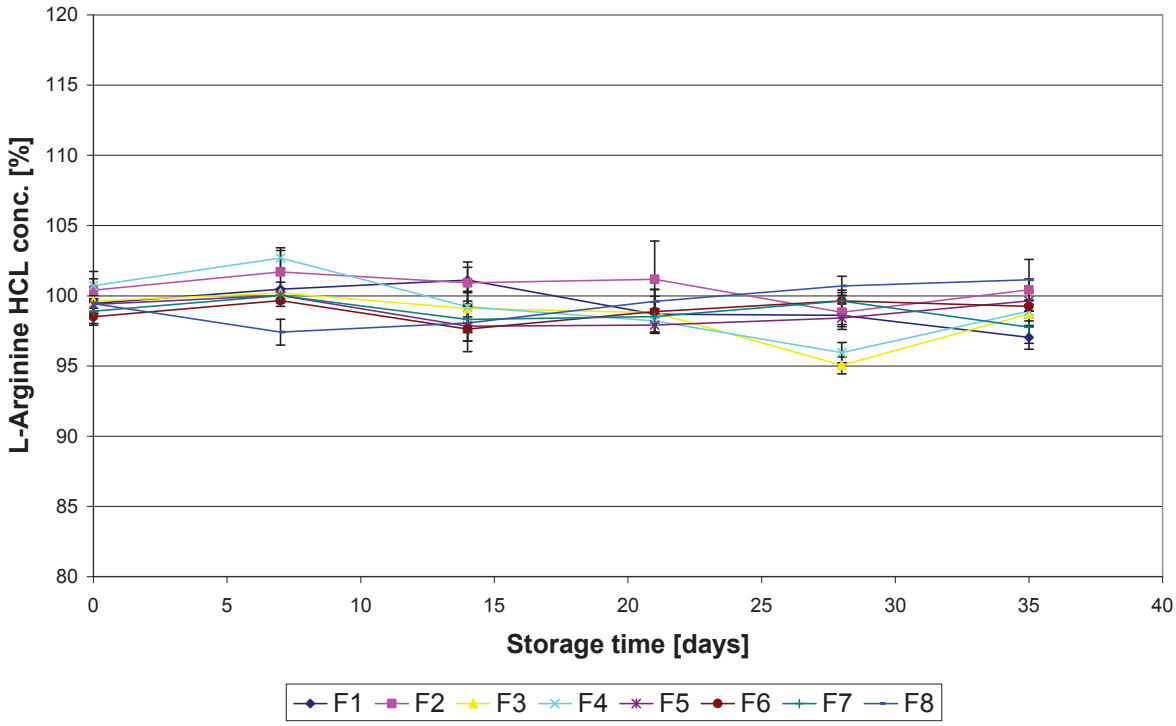

Figure 5: L-arginine HCL concentrations during first preformulation stability study, storage at 5°C determined by HPLC (n = 3; mean ± SD).

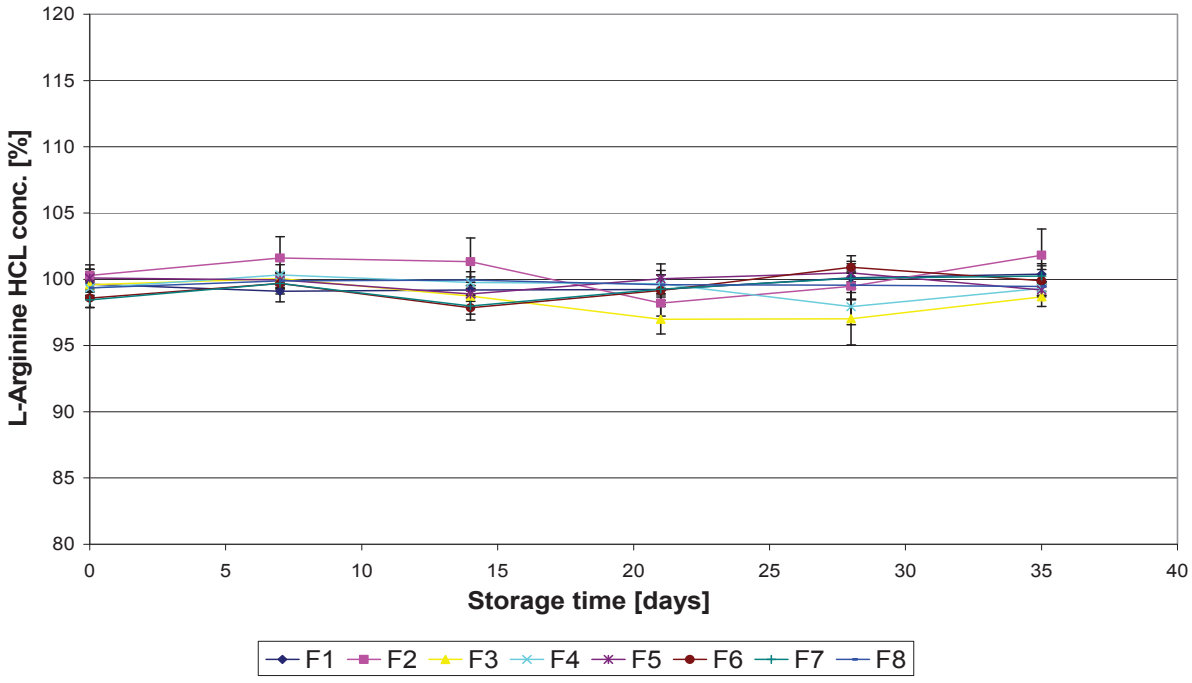

Figure 6: L-arginine HCL concentrations during first preformulation stability study, storage under room temperature at 25°C determined by HPLC (n = 3; mean ± SD).

The presence of sodium bisulfite and EDTA in the formulation, and the pH of the solution adjusted to 6.5, stabilized L-arginine HCL, but the absence of sodium bisulfite and disodium EDTA in the formulation, and the pH of the solution adjusted to 6.5 did not stabilize L-arginine HCL. The presence of glycerin in the formulation has no effect on the stability of L-arginine HCL. The concentration of L-arginine HCL decreased in the formulations F1 and F3 containing no sodium bisulfite and disodium EDTA, figure 7.

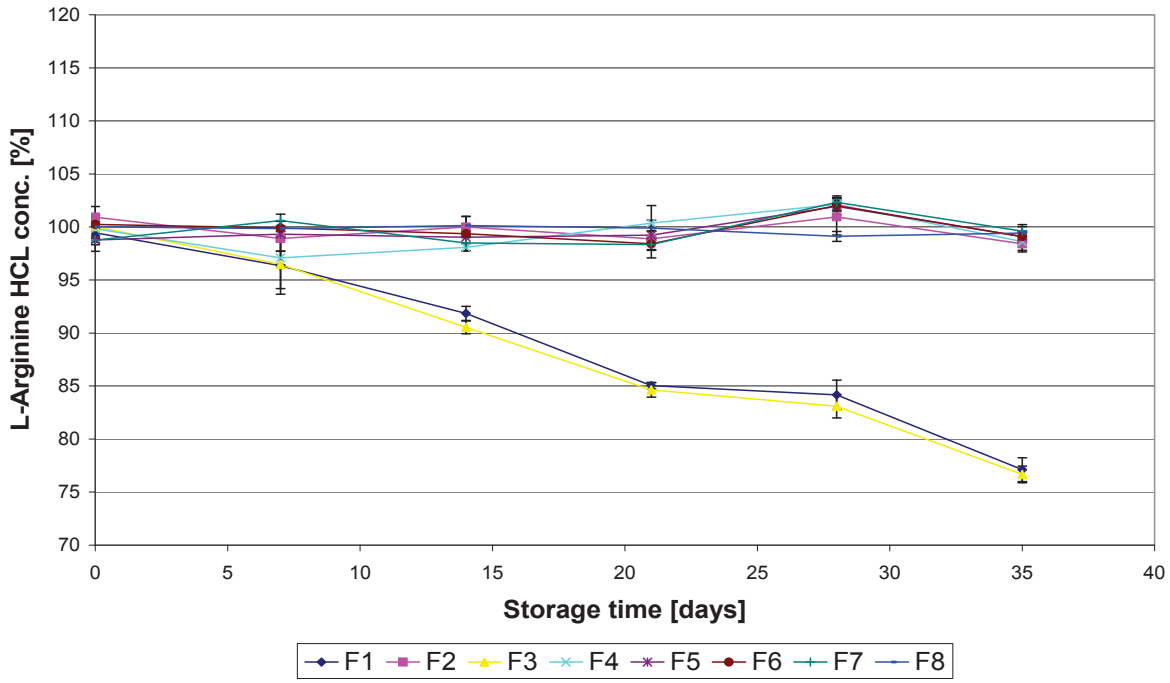

Figure 7: L-arginine HCL concentrations during first preformulation stability study, storage under accelerated conditions at 40°C determined by HPLC (n = 3; mean ± SD).

After 35 days, only 77.11% and 76.66% of L-arginine HCL was found in the formulations F1 and F3 respectively (table 6) that stored at 40°C. In other formulations F2, F4, F5, F6, F7 and F8 that stored at 40°C, the L-arginine content was within the standard range, this mean the higher temperature 40°C accelerates the oxidation and degradation of L-arginine in the formulations (F1 and F3) that containing no sodium bisulfite and disodium EDTA, see methods presented 2.2.1.5.2 section (stress tests), which match the results of studies.

Table 6: Percent of L-arginine HCL remaining in each formulation after 35 days, stored at different temperatures (n = 3; mean ± SD).

Stored temperature	**F1**	**F2**	**F3**	**F4**
5°C	99.23±0.81	100.57±1.42	98.58±0.77	99.28±0.93
25°C	99.60±0.91	100.45±1.31	98.50±0.90	99.38±0.77
40°C	77.11±0.63	99.67±0.77	76.66±0.78	99.35±0.79
Stored temperature	**F5**	**F6**	**F7**	**F8**
5°C	98.86±0.74	98.92±0.84	98.85±0.79	99.39±1.01
25°C	99.77±0.79	99.34±0.86	99.28±0.49	99.62±0.69
40°C	99.56±0.59	99.83±0.46	99.68±0.71	99.73±0.75

To evaluate the presence or absence of each factor influencing L-arginine stability, the full factorial block was conducted. Table 7 demonstrates average percent L-arginine remaining with each factor after 35 days of storage at different temperature. The data are from table 6. For example, to examine the presence of disodium EDTA in the preparation storage under accelerated conditions at 40°C, it can be achieved by obtaining the average value (L-arginine concentration) under the glycerin + block (table 4). These values are 99.35 (F4), 99.56 (F5), 99.68 (F7) and 99.73 (F8). The resulting average value is therefore 99.58. On the other hand, the value in the formulation with the absence of disodium EDTA is 88.31.

Table 7: Percent of L-arginine HCL remaining from the effect of each factor during first preformulation stability, storage at different temperatures (n=4).

Factors		Mean ± SD at 5°C	Mean ± SD at 25°C	Mean ± SD at 40°C
Sodium bisulfite	-	98.98±0.33	99.08±0.49	88.20±0.20
	+	99.43±0.79	99.79±0.47	99.69±0.11
Glycerin	-	99.48±0.74	99.57±0.66	96.89±0.27
	+	98.93±0.33	99.18±0.48	96.95±0.58
Disodium EDTA	-	99.32±0.87	99.47±0.80	88.31±0.33
	+	99.09±0.28	99.28±0.31	99.58±0.16

Thus, in this case the presence of disodium EDTA in the formulation was preferred as it gave a higher average value (L-arginine remaining) than that of the absence. A similar procedure was applied to the other factors.

Continuing the analysis indicated that the presence of disodium EDTA and sodium bisulfite, in addition to the pH of the solution adjusted to 6.5, stabilized L-arginine in the solution system storage under accelerated conditions at 40°C.

No precipitation was observed in any of the samples during the storage period. The pH of F1 and F3 was reasonably stable (table 10) stored at 40°C. The pH of F2, F4, F5, F6, F7 and F8 was fairly constant after 35 days of storage at 5°C, 25°C and 40°C (tables 8, 9, 10). The change in the pH of F1 and F3 were found to relate to the decrease of L-arginine concentration.

Table 8: pH of L-arginine HCL (first preformulation study), storage under room temperature at 25°C (n = 3; mean ± SD).

Day	**F1**	**F2**	**F3**	**F4**
0	6.50±0.00	6.50±0.05	6.52±0.08	6.48±0.02
7	6.49±0.05	6.48±0.06	6.51±0.06	6.50±0.00
14	6.54±0.12	6.48±0.03	6.48±0.02	6.50±0.00
21	6.51±0.07	6.47±0.06	6.48±0.02	6.49±0.01
28	6.51±0.12	6.50±0.08	6.50±0.00	6.52±0.04
35	6.52±0.06	6.50±0.00	6.50±0.11	6.51±0.06
Day	**F5**	**F6**	**F7**	**F8**
0	6.50±0.04	6.51±0.11	6.50±0.00	6.51±0.06
7	6.53±0.10	6.50±0.00	6.45±0.08	6.50±0.08
14	6.51±0.07	6.51±0.03	6.50±0.00	6.51±0.06
21	6.50±0.00	6.51±0.07	6.50±0.07	6.52±0.04
28	6.51±0.11	6.48±0.10	6.52±0.04	6.51±0.03
35	6.51±0.13	6.50±0.00	6.48±0.03	6.47±0.08

Table 9: pH of L-arginine HCL (first preformulation study), storage at 5°C (n = 3; mean ± SD).

Day	**F1**	**F2**	**F3**	**F4**
0	6.50±0.00	6.52±0.06	6.50±0.05	6.48±0.05
7	6.51±0.03	6.50±0.08	6.53±0.10	6.51±0.11
14	6.48±0.10	6.52±0.06	6.51±0.07	6.54±0.07
21	6.51±0.11	6.50±0.00	6.54±0.10	6.50±0.04
28	6.50±0.00	6.51±0.06	6.51±0.06	6.51±0.09
35	6.50±0.07	6.51±0.07	6.48±0.10	6.50±0.00
Day	**F5**	**F6**	**F7**	**F8**
0	6.50±0.03	6.49±0.07	6.48±0.09	6.50±0.00
7	6.51±0.03	6.52±0.08	6.51±0.07	6.52±0.05
14	6.50±0.03	6.50±0.00	6.50±0.05	6.51±0.04
21	6.51±0.60	6.48±0.03	6.50±0.00	6.50±0.06
28	6.50±0.00	6.48±0.02	6.50±0.11	6.47±0.11
35	6.50±0.05	6.51±0.07	6.53±0.08	6.51±0.09

Table 10: pH of L-arginine HCL (first preformulation study), storage under accelerated conditions at 40°C (n = 3; mean ± SD).

Day	F1	F2	F3	F4
0	6.50±0.06	6.50±0.05	6.50±0.00	6.48±0.10
7	6.27±0.09	6.57±0.10	6.30±0.07	6.50±0.05
14	6.04±0.04	6.52±0.10	5.99±0.06	6.50±0.05
21	5.62±0.06	6.50±0.00	5.59±0.05	6.52±0.09
28	5.51±0.05	6.52±0.04	5.48±0.04	6.50±0.05
35	5.10±0.03	6.48±0.07	5.05±0.04	6.48±0.06
Day	**F5**	**F6**	**F7**	**F8**
0	6.52±0.08	6.50±0.00	6.49±0.05	6.50±0.00
7	6.51±0.09	6.53±0.16	6.46±0.08	6.48±0.13
14	6.53±0.13	6.50±0.07	6.50±0.07	6.51±0.10
21	6.48±0.06	6.51±0.09	6.47±0.10	6.56±0.09
28	6.49±0.01	6.50±0.00	6.50±0.05	6.50±0.05
35	6.51±0.09	6.50±0.00	6.45±0.08	6.49±0.04

From the preformulation study, it can be summarized here that pH 6.5 is the optimum pH for L-arginine stability in solution system and the presence of disodium EDTA and sodium bisulfite improved L-arginine stability at a higher temperature 40°C.

The formulation F4 will be the master formula for the formulation development of 10% L-arginine HCL solution, since it had the same stability as those formulations content sodium bisulfite. Sodium bisulfite has been found to cause allergic reaction in paediatric formulation [19, 20, 21, 22].

1.5 Second preformulation study

The master formula of second preformulation study was L-arginine solution consisted of 10% (w/v) L-arginine HCL, 0.01% (w/v) domiphenbromide as a preserving agent, 1% (w/v) disodium EDTA as a chelating agent and purified water. The master formula of second preformulation study was selected from the first preformulation study. In this study, 3 factors cherry syrup, raspberry syrup and honey were chosen to be evaluated. The total number of the experimental formulations was 5 (see table 11).

Table 11: Five formulations of 10% L-arginine HCL solution in second preformulation study (n=3).

Components	F1 % w/v	F2 % w/v	F3 % w/v	F4 % w/v	F5 % w/v
L-arginine HCL	10	10	10	10	10
Domiphenbromide	0.01	0.01	0.01	0.01	0.01
Disodium EDTA	0.1	0.1	0.1	0.1	0.1
Sucralose	0.05	0.05	0.05	0.05	0.05
Cherry syrup	20	-	-	10	-
Honey	-	10	-	5	5
Raspberry syrup	-	-	20	-	10
Banana flavour	0.02	0.02	-	0.02	0.02
Orange flavour	-	-	0.02	-	0.02
Purified water (ml) to	100	100	100	100	100
pH adjusted to	6.5	6.5	6.5	6.5	6.5

Cherry syrup, raspberry syrup and honey were added (if presented) at concentrations of 20% (w/v), 20% (w/v) and 10% (w/v), respectively. Three bottles of each formulation were prepared and kept in amber glass bottles (n=3), stored at 5°C, 25°C and 40°C for 35 days.

Second prefomulation approach was employed for the formulation development and stability testing of L-arginine HCL 10% solution. Factors expecting to affect the stability of L-arginine HCL were evaluated, i.e., cherry syrup, raspberry syrup and honey. Five formulations were prepared. The stability of the preparations was tested after 35 days of storage. The data of second preformulation study will be use for formulation development. The formulations from F1 to F5 that stored at 5°C, 25°C and 40°C were stable and within the standard range of L-arginine content (97-103%), as seen in figures 8, 9, 10.

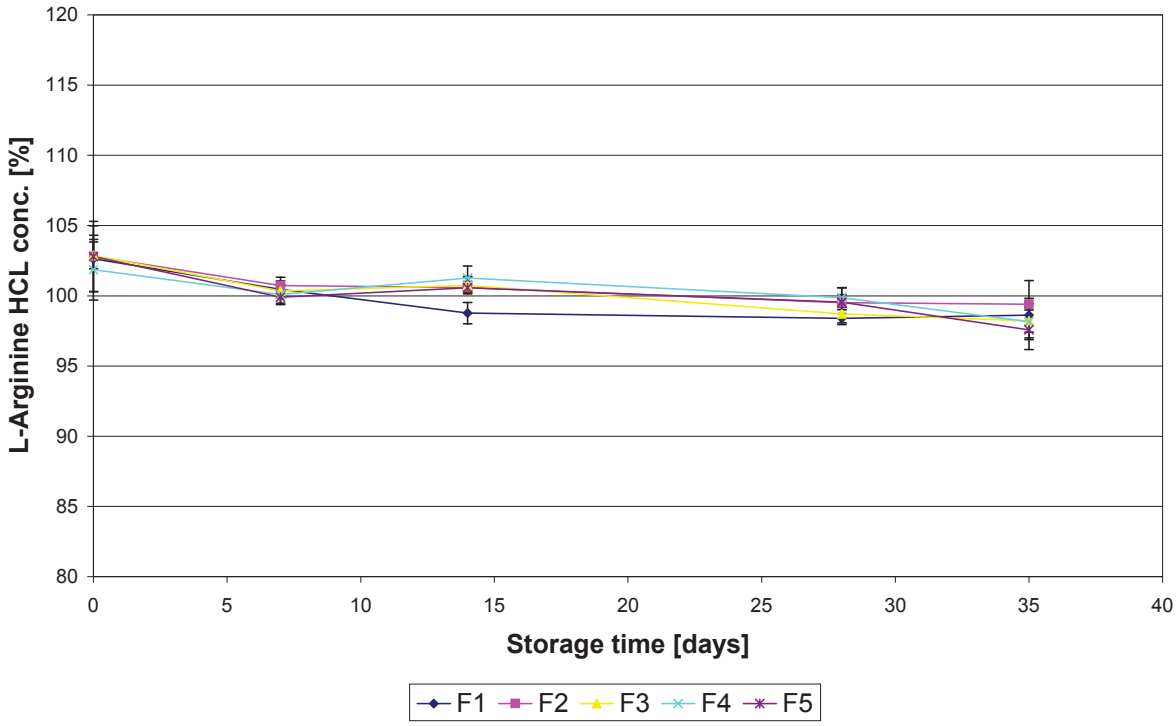

Figure 8: L-arginine HCL concentrations during second preformulation stability study, storage at 5°C determined by HPLC (n = 3; mean ± SD).

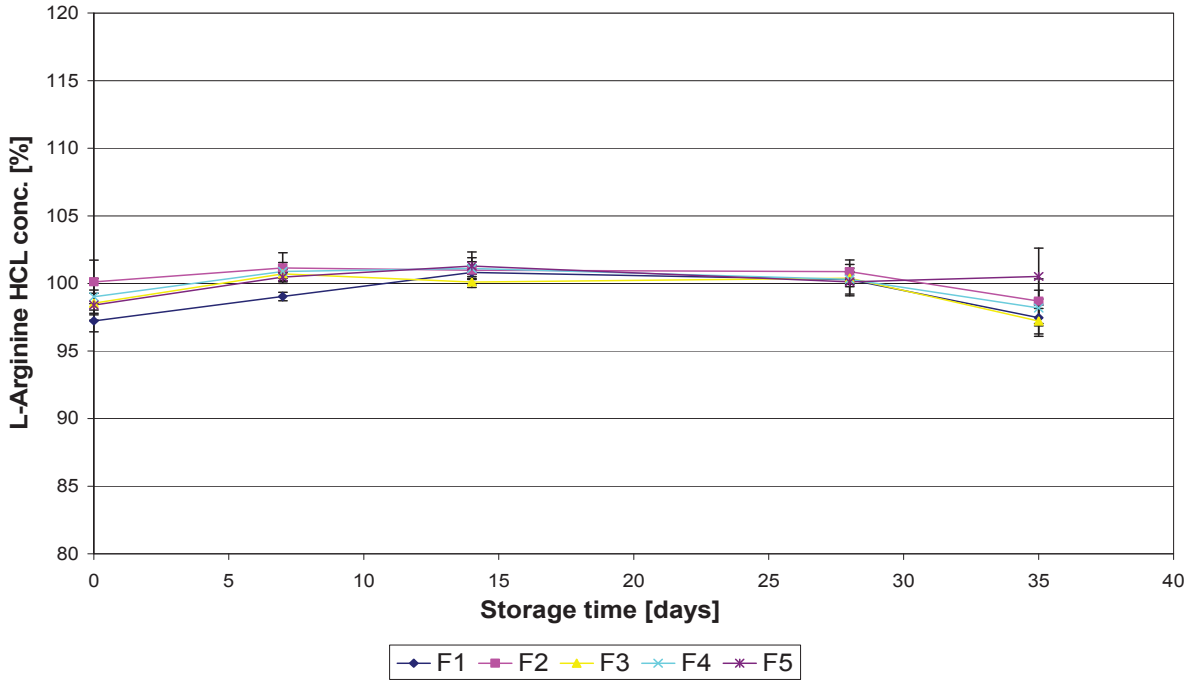

Figure 9: L-arginine HCL concentrations during second preformulation stability study, storage under room temperature at 25°C determined by HPLC (n = 3; mean ± SD).

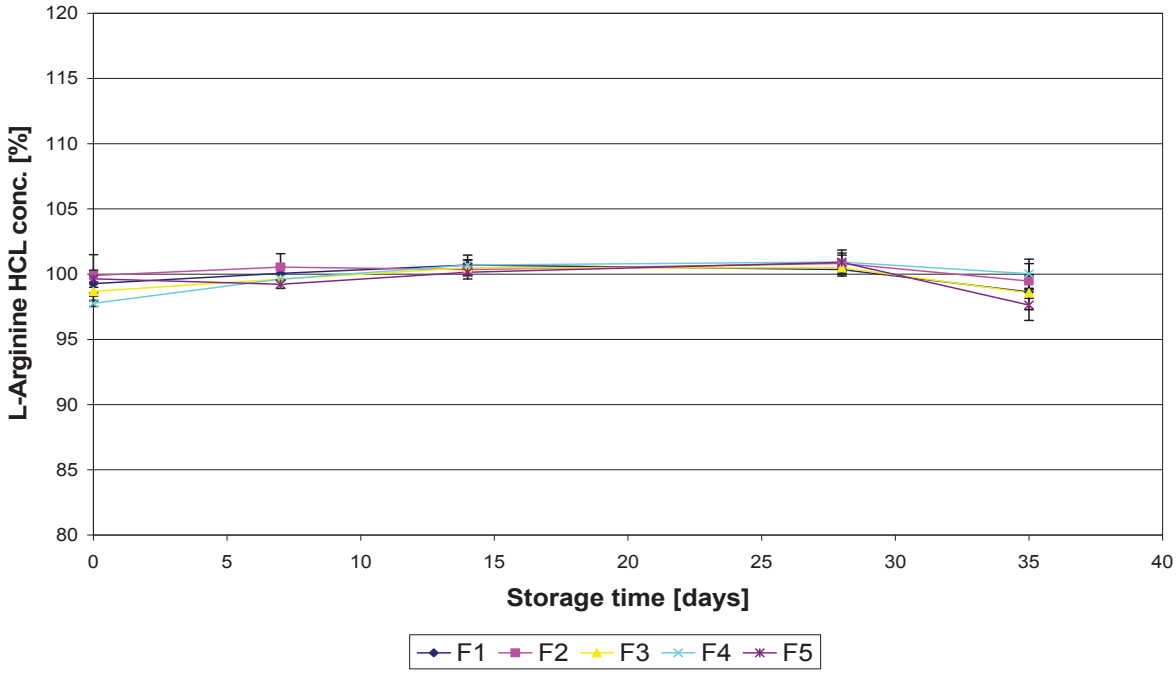

Figure 10: L-arginine HCL concentrations during second preformulation stability study, storage under accelerated conditions at 40°C determined by HPLC (n = 3; mean ± SD).

The presence of cherry syrup, raspberry syrup and honey in the formulation, and the pH adjusted to 6.5, did not affect the stability of L-arginine HCL (table 12). No precipitation was observed in any of the samples during the storage period. The pH of the five formulations was constant after 35 days of storage at 5°C, 25°C and 40°C, see tables 13, 14, 15.

Table 12: Percent of L-arginine remaining of each formulation after 35 days, stored at different temperatures (n = 3; mean ± SD).

Stored temperature	F1	F2	F3	F4	F5
5°C	99.77±1.04	100.60±1.12	100.17±0.76	100.24±1.14	100.07±1.06
25°C	98.96±0.71	100.36±1.17	99.39±0.57	99.89±1.13	100.15±1.04
40°C	99.80±0.69	100.22±1.06	99.56±0.63	99.80±0.68	99.51±0.63

Table 13: pH of L-arginine HCL (second preformulation study), storage at 5°C (n = 3; mean ± SD).

Day	F1	F2	F3	F4	F5
0	6.47±0.04	6.52±0.06	6.48±0.03	6.52±0.08	6.53±0.05
7	6.51±0.09	6.52±0.06	6.50±0.04	6.50±0.01	6.48±0.07
14	6.51±0.07	6.48±0.05	6.51±0.05	6.49±0.07	6.50±0.03
28	6.49±0.03	6.48±0.02	6.51±0.04	6.52±0.03	6.50±0.04
35	6.49±0.06	6.51±0.04	6.49±0.03	6.51±0.03	6.52±0.08

Table 14: pH of L-arginine HCL (second preformulation study), storage under room temperature at 25°C (n = 3; mean ± SD).

Day	F1	F2	F3	F4	F5
0	6.53±0.04	6.49±0.03	6.50±0.05	6.48±0.03	6.50±0.05
7	6.51±0.06	6.52±0.05	6.49±0.03	6.49±0.06	6.50±0.04
14	6.50±0.04	6.51±0.04	6.48±0.03	6.50±0.02	6.49±0.06
28	6.50±0.05	6.48±0.02	6.51±0.06	6.50±0.01	6.50±0.05
35	6.49±0.03	6.50±0.03	6.50±0.01	6.51±0.02	6.50±0.05

Table 15: pH of L-arginine HCL (second preformulation study), storage under accelerated conditions at 40°C (n = 3; mean ± SD).

Day	F1	F2	F3	F4	F5
0	6.51±0.03	6.51±0.03	6.49±0.03	6.50±0.04	6.51±0.04
7	6.50±0.06	6.51±0.05	6.50±0.02	6.48±0.05	6.50±0.06
14	6.49±0.03	6.50±0.05	6.48±0.03	6.50±0.01	6.48±0.02
28	6.51±0.04	6.50±0.02	6.49±0.03	6.51±0.02	6.50±0.05
35	6.51±0.06	6.49±0.05	6.51±0.02	6.48±0.02	6.51±0.06

From the second preformulation study, it can be summarized here that pH 6.5 is the optimum pH for L-arginine HCL stability in solution system. The presence of cherry syrup, raspberry syrup and honey in the formulations does not affect the stability of L-arginine HCL and the formulations.

1.6 Long-term stability study

Three bottles of each formulation were prepared and kept in amber glass bottles (n=3), stored at 5°C, 25°C and 40°C for 12 months, based on the results obtained from the first and second preformulation studies. L-arginine HCL was first dissolved in a part of purified water. Ingredients as listed in each formulation were added and mixed using a magnetic stirrer, followed by pH and volume adjustment. It was found that clear colour solutions were observed. The color of the samples was slightly yellowish and pinkish. The stability of these samples was investigated because oxidation was assessed to be major factor influencing L-arginine HCL stability. Disodium EDTA was added to all formulations as an antioxidant and chelating agent.

After several preliminary trials cherry syrup, raspberry syrup, honey and two different flavours at concentrations as shown in table 11 were added for taste masking of L-arginine HCL. The mass of delivered doses from multidose containers of 10% L-arginine HCL solution was uniform. The osmolarity of formulations was under 1900 mosmol/kg and this is an adequate and acceptable range of osmolarity for paediatric use was reached by using different masking agents [145].
No precipitation and color change were observed in any of the samples during the 12 months of storage at different storage conditions. The taste was the same throughout the study period. A decrease of odour was observed in all samples after 9 months of storage at 40°C. A slight change of pH was observed in all samples, it can be therefore concluded here that the buffering agent used was capable of controlling the pH of the solution. In addition, the reasonably constant pH of all formulations was related to L-arginine stability as higher than 97% of the drug was found (tables 17, 18, 19).

The formulations from F1 to F5 that stored at 5°C, 25°C and 40°C were stable and within the standard range of L-arginine content (97-103%), as can be seen in table 16 and figures 11, 12, 13.

Table 16: Percent L-arginine remaining of each formulation after 12 months, stored at different temperatures (n = 3; mean ± SD).

Stored temperature	F1	F2	F3	F4	F5
5°C	99.29±1.47	99.60±1.19	98.89±1.11	99.22±1.18	99.84±1.59
25°C	99.26±1.22	98.95±1.10	99.53±1.15	99.15±0.89	98.33±1.03
40°C	98.47±1.06	98.77±1.43	99.67±0.92	98.86±0.80	98.33±1.09

Table 17: pH of L-arginine HCL (Long-term stability study), storage at 5°C (n = 3; mean ± SD).

month	F1	F2	F3	F4	F5
0	6.47±0.04	6.52±0.06	6.08±0.03	6.52±0.08	6.53±0.05
3	6.50±0.05	6.53±0.06	6.48±0.05	6.50±0.03	6.49±0.07
6	6.48±0.06	6.50±0.00	6.49±0.03	6.50±0.00	6.51±0.06
9	6.48±0.06	6.50±0.05	6.51±0.09	6.50±0.00	6.48±0.02
12	6.51±0.03	6.50±0.05	6.51±0.04	6.53±0.07	6.50±0.05

Table 18: pH of L-arginine HCL (Long-term stability study), storage under room temperature at 25°C (n = 3; mean ± SD).

month	F1	F2	F3	F4	F5
0	6.53±0.04	6.49±0.03	6.50±0.05	6.48±0.03	6.50±0.05
3	6.51±0.06	6.47±0.04	6.48±0.03	6.50±0.00	6.52±0.04
6	6.49±0.06	6.50±0.02	6.50±0.05	6.48±0.06	6.50±0.03
9	6.48±0.03	6.51±0.04	6.50±0.00	6.50±0.01	6.48±0.07
12	6.50±0.05	6.49±0.01	6.50±0.00	6.51±0.03	6.50±0.00

Table 19: pH of L-arginine HCL (Long-term stability study), storage under accelerated conditions at 40°C (n = 3; mean ± SD).

month	F1	F2	F3	F4	F5
0	6.51±0.03	6.51±0.03	6.49±0.03	6.50±0.04	6.51±0.04
3	6.50±0.07	6.50±0.03	6.49±0.05	6.51±0.03	6.49±0.01
6	6.50±0.07	6.50±0.00	6.48±0.03	6.50±0.08	6.49±0.01
9	6.52±0.06	6.51±0.05	6.50±0.00	6.51±0.07	6.52±0.06
12	6.50±0.05	6.50±0.00	6.52±0.05	6.53±0.07	6.50±0.03

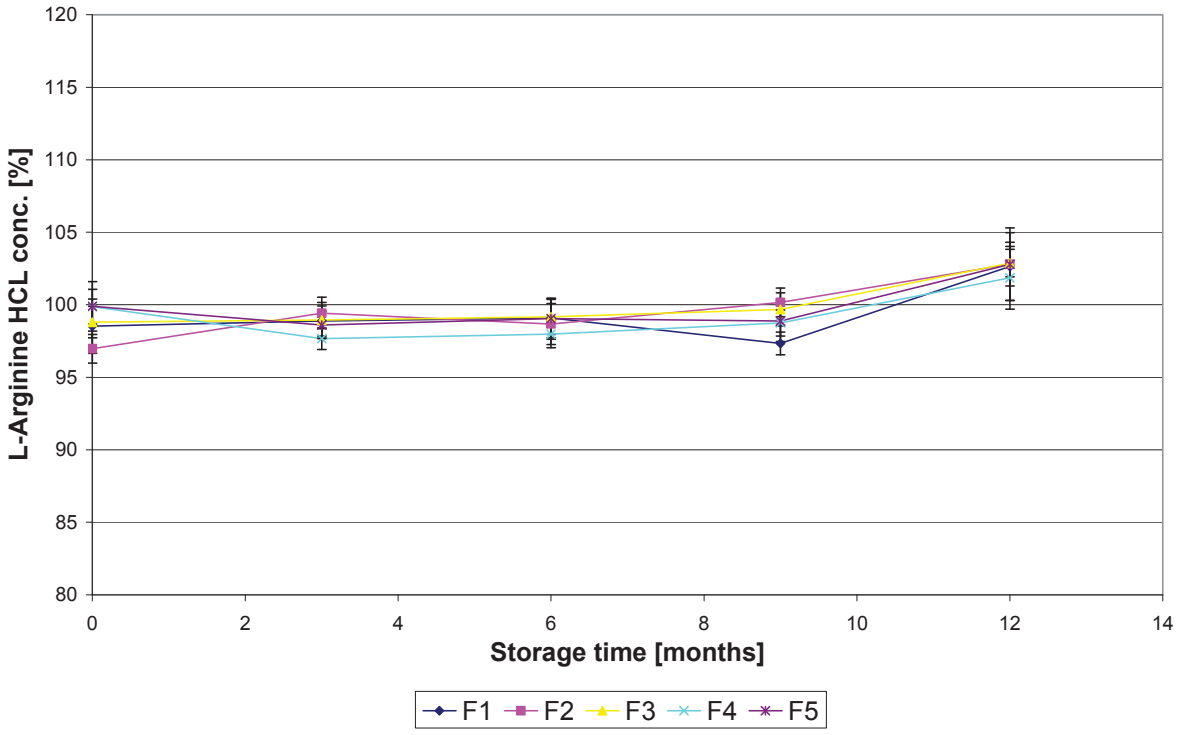

Figure 11: L-arginine HCL concentrations during long-term stability study, storage at 5°C determined by HPLC (n = 3; mean ± SD).

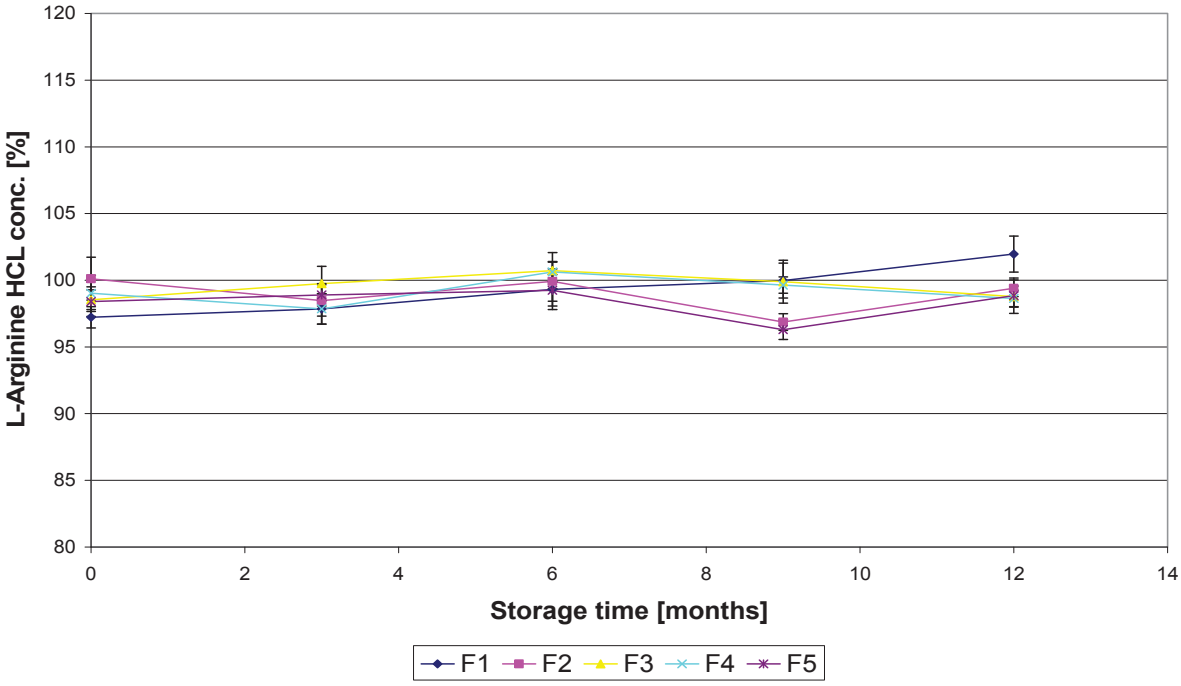

Figure 12: L-arginine HCL concentrations during long-term stability study, storage under room temperature at 25°C determined by HPLC (n = 3; mean ± SD).

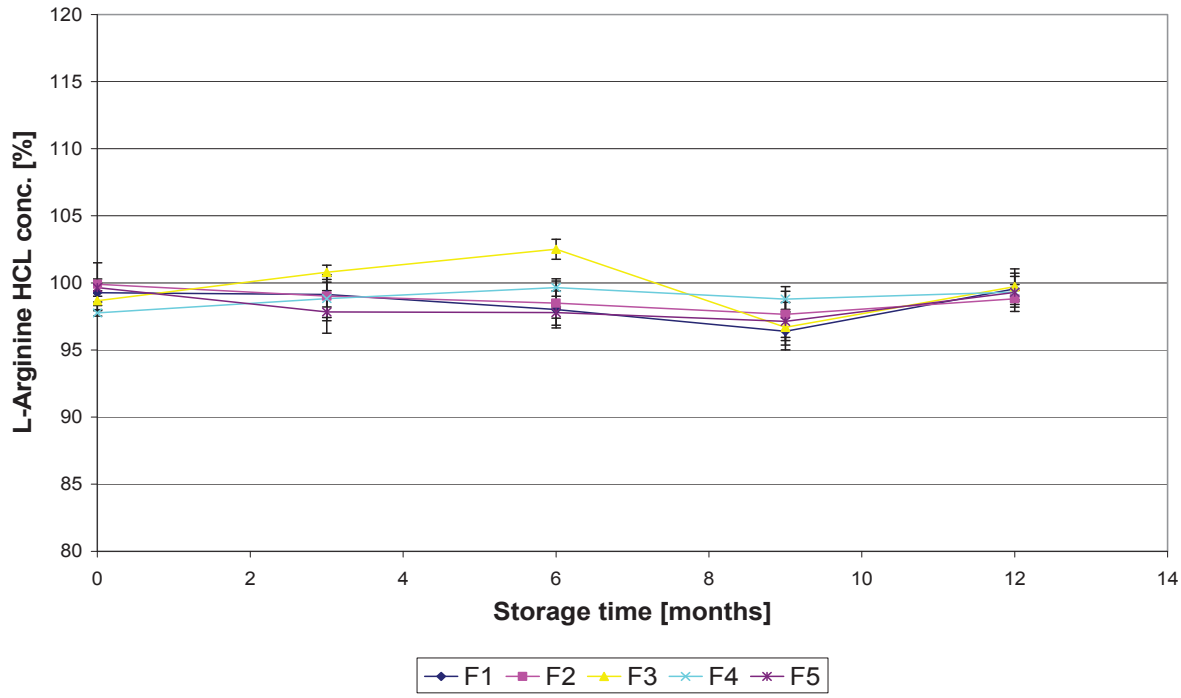

Figure 13: L-arginine HCL concentrations during long-term stability study, storage under accelerated conditions at 40°C determined by HPLC (n = 3; mean ± SD).

1.7 In-use stability test

Medicinal products in multidose containers, which by nature of their physical form and chemical composition due to repeated opening and closing, may pose a risk to its content with regard to microbiological contamination, proliferation and/or physicochemical degradation once the closure system has been breached. The continued integrity of products in multidose containers after the first opening is an important quality issue.

The purpose of in use stability testing is to establish, where applicable, a period of time during which a multidose product may be used following the removal of the appropriate doses from the container without adversely affecting the integrity of the product. Such testing is applicable to all preparations supplied in multidose containers. However, it is particularly important that such testing is also applied if the formulation includes an antioxidant or an antimicrobial preservative. Testing may also be applicable to multidose oral or topical dosage forms if a specific problem in terms of stability of the product has been identified, once the container has been opened. The situation would apply for example, to an oral product in which the active ingredient, which is subject to oxidative degradation, has been packed in a well filled, oxygen-impermeable container. The testing procedure involves the removal of aliquots as defined under test protocol, of the product from the container at regular intervals and the determination of physical and chemical properties of the product over the period of the proposed in in-use stability test [17]. While this principle is acknowledged in the Ph. Eur. and EU guidelines, no specific guidance is available on defining test design and conduct of studies to be undertaken to define in-use stability test in a uniform fashion [18].

Three bottles of each formulation were stored in amber glass bottles (n=3), for 60 days at different storage conditions (table 20). During the first 60 days, 1.5 mL was daily removed as an in-use sample. Sampling was performed under normal environmental conditions of use. The test parameters used in the in-use stability test of 10% L-arginine HCL solution were physical (colour, clarity, odour and pH) and chemical properties (active substance assay).

Testing performed at intermediate time points and at the end of the proposed in-use stability test on the final remaining amount of the product in the container. There was no change in colour and odour of the formulations, no precipitation was observed visually in any of the samples during the storage period. The stability of the preparations was tested after 60 days of storage. The formulations were stable and within the specified range of L-arginine content (97-103%), as can be seen in table 21 and figure 14. The pH of the formulations was constant (table 22).

Table 20: Testing points and storage conditions for in-use stability evaluation.

Storage temperature	25°C/60% RH	5°C/40% RH	40°C/75% RH
Sample/Time (days)	0, 10, 40	20, 50	30, 60

Table 21: Percent of L-arginine remaining of each formulation after 60 days, stored under in-use conditions (n = 3; mean ± SD).

Sample/ Time(day)	F1	F2	F3	F4	F5
0	101.81±1.90	97.05±1.97	99.19±1.85	97.99±1.85	97.12±1.87
10	99.39±1.41	98.33±2.02	98.83±1.30	98.97±2.30	101.44±1.84
20	99.81±1.74	101.64±1.94	100.20±1.96	100.08±1.31	99.05±1.59
30	100.98±0.95	100.50±1.62	98.52±1.34	100.89±1.45	100.00±1.89
40	101.77±1.19	102.71±1.56	101.47±1.54	101.72±1.60	101.41±1.54
50	100.43±1.56	100.25±2.12	99.12±1.48	100.13±1.94	100.42±1.37
60	97.33±0.30	99.61±0.87	101.07±0.62	101.17±1.22	99.58±1.09

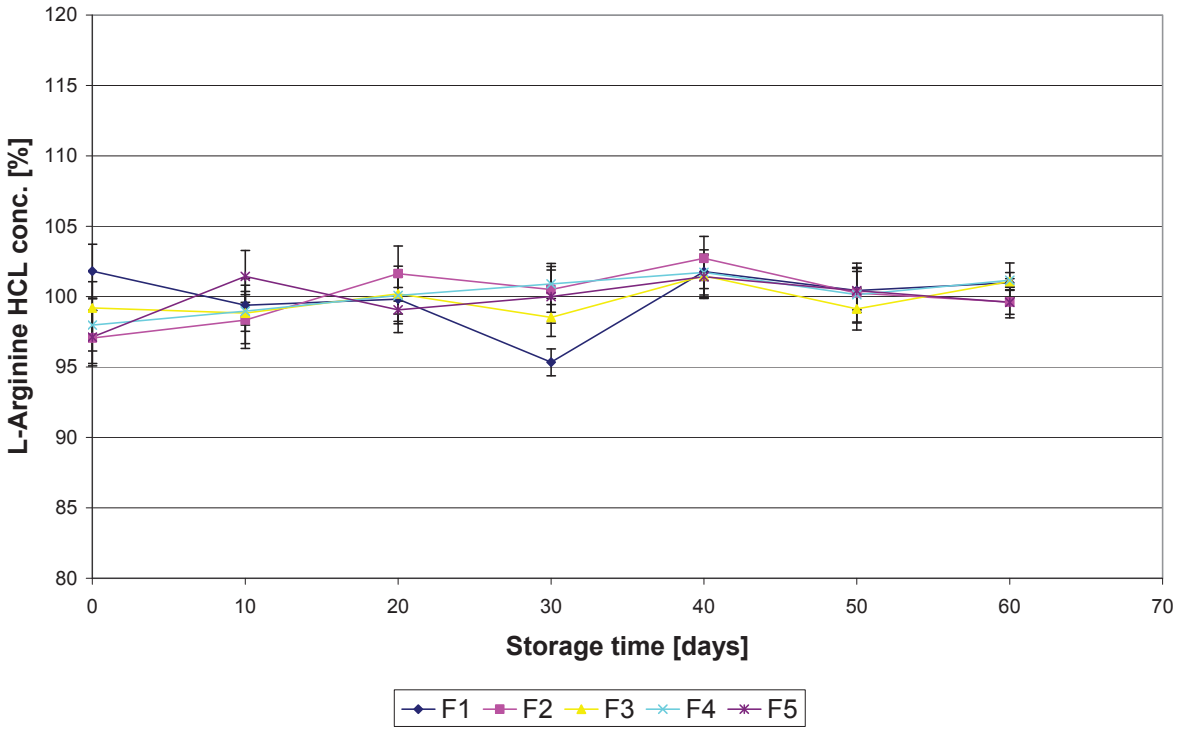

Figure 14: L-arginine HCL concentrations during In-use stability test of 10% L-arginine HCL solutions, stored under in-use conditions (n = 3; mean ± SD).

Table 22: pH of L-arginine HCL (In-use stability test), stored under in-use conditions (n = 3; mean ± SD).

Days	F1	F2	F3	F4	F5
0	6.52±0.06	6.50±0.12	6.52±0.08	6.48±0.11	6.50±0.00
10	6.53±0.05	6.47±0.04	6.50±0.04	6.49±0.05	6.52±0.04
20	6.51±0.07	6.50±0.05	6.52±0.06	6.48±0.06	6.50±0.05
30	6.50±0.08	6.50±0.00	6.54±0.07	6.52±0.04	6.51±0.03
40	6.48±0.07	6.50±0.05	6.51±0.03	6.49±0.01	6.50±0.07
50	6.47±0.06	6.53±0.05	6.50±0.00	6.48±0.09	6.50±0.05
60	6.50±0.01	6.52±0.04	6.51±0.03	6.48±0.06	6.49±0.03

1.8 Conclusion

This study demonstrates that the first preformulation study using the full factorial block design can be used successfully to evaluate the factors affecting L-arginine HCL stability. The pH 6.5 was the optimum pH for L-arginine HCL stability in solution system and the presence of disodium EDTA and sodium bisulfite assisted L-arginine HCL stability at a higher temperature 40°C. The formulation F4 was selected as the master formula for the subsequent formulation development of 10% L-arginine HCL solution because it had the same stability as those formulations containing sodium bisulfite. Sodium bisulfite has been found to give allergic reaction in pediatric patients and have been associated with a number of severe to fatal adverse reactions [23], although it is extensively used in a variety of preparations. Therefore, it is beneficial if its use can be restricted. The second preformulation study was used to minimize the number of taste masked formulations by using masking agents for further long-term stability.

Other ingredients added such as cherry syrup, raspberry syrup, honey and two different flavours did not affect the stability of L-arginine, and in fact, improved the taste. It can be concluded that these 5 formulations of L-arginine HCL solution were stable and can be kept at the 5°C, 25°C and 40°C for at least 12 months. We provide evidence for storage and in-use stability of at least 5 formulations of 10% L-arginine HCL.

2. Multiparticulate formulations

2.1 Extrusion/spheronization process

2.1.1 Introduction

Pellets are spherical granules of varying diameter depending on the application and the intention of the developer [26]. Applications are found not only in the pharmaceutical industry but also in the food industry (e.g., fish food) and in the chemical industry. Pellets as a drug delivery system offer not only therapeutic advantages such as less irritation of the gastro-intestinal tract and a lowered risk of side effects due to dose dumping, but also technological advantages, for example, better flow properties, less friable dosage form, narrow particle size distribution, ease of coating and uniform packing. The reproducibility of drug blood levels is an additional advantage to the use of a pellet formulation. Pellets, manufactured in the pharmaceutical industry, are sized commonly between 500 and 1500 μm [26]. They may be filled into hard gelatine capsules but can also be compressed to tablets or in the case of paediatric drug delivery, small pellets can be given to small children by sprinkling them on food. Pellets are the solid dosage form of choice for peroral administration to the children over 2 years old in comparing to monolithic dosage forms [5]. Pellets can be produced in different ways, but the most popular method of producing pellets is by the extrusion-spheronisation technique. The extrusion process is the shaping or pressing of a wet mass or plasticize a material in to long rods though defined openings during extrusion.

The extrusion process can be performed using four main classes of extruders: screw, sieve and basket, roll, and ram extruders. The screw extruder consists of one or two screws feeding the plastic mass to an axial or radial extrusion screen. In the axial type, the screen is placed at the end of the screw, perpendicularly with the axis of the screw in contrast to the radial type where the die is placed around the screw, discharging the extrudate perpendicularly to the axis of the screw [26].

Wet extrusion involves four steps: preparation of the wet mass (granulation), shaping the wet mass into cylinders (extrusion), breaking up the extrudate and rounding of the particles into spheres (spheronisation) and finally drying of the pellets, the last step is the critical step in the wet extrusion, depending on the drying method used, the porosity, surface structure and particle size of the pellets may be affected [27, 28, 29, 30]. The present work focuses on the development of spherical multiparticulate systems by the extrusion-spheronisation process.

Melt extrusion processes are currently applied in the pharmaceutical field for the manufacture of a variety of dosage forms and formulations such as granules, pellets, tablets, suppositories, implants, stents, transdermal systems and ophthalmic inserts. As a specific area the manufacture of solid dispersions. Melt extrusion is considered to be an efficient technology in this field with particular advantages over solvent processes like co-precipitation. With regard to pharmaceuticals, most systems extruded today consist of particles dispersed in a matrix, where matrix is present between solid particles. The relative position of solids can change during the various stages of the extrusion process [31]. The production of pellets by extrusion at the room temperature (cold extrusion) becomes possible on the basis of lipids [50]. The applicability of the solid lipid extrusion process as preparation method for sustained release dosage forms [33], and for immediate release dosage forms [51], was investigated and described.

Spheronization is a rapid and flexible process where pharmaceutical products are formed into small spheres, or spheroids for producing spherical agglomerates that have many technological and therapeutical advantages. Rotary processing is an efficient multistage, single-pot spheroids production method. The rotary processor can be used for spheroids production, drying as well as coating. In the course of spheroids production, centrifugal, fluidizing, and gravitational forces act upon the product from different directions and collectively contribute to the spheroid formation process during rotary processing. The outcome of the process depends on the complex interactions between the equipment, formulation, and process variables [34].

2.2 Wet extrusion

2.2.1 MCC and HPMC

The formulation aids available for pelletization by extrusion/spheronization are very limited due to the characteristics desired from their wet masses. The wet mass must meet the requirements for both extrusion, as well as spheronization. The main requirement for the extrusion process is the ability of the powder material to form a cohesive plastic mass upon wetting that remains homogenous throughout the extrusion process. For spheronization, a balance between plasticity and brittleness of the wet mass is needed to successfully obtain the product. Since rheological behaviour of the wet powder mass, which is very important for both extrusion and spheronization processes, is dependent on the physical properties of the powders, such as particle size, size distribution, density, surface area and morphology, the physical properties of the powder materials play a critical role in obtaining a pellet product with desired attributes [41].

Cellulose qualities are the most important excipients for extrusion/spheronization process. Usually, cellulose is produced from wood by washing, bleaching, purifying, and drying. Powdered cellulose (PC) is generally obtained by mechanically micropulverizing cellulose. It has a high amount of amorphous regions and is often used as filler and binder in tablet manufacture. Microcrystalline cellulose (MCC) has a higher degree of crystallinity because it is usually obtained by partially hydrolyzing cellulose with mineral acid.

Subsequently, porous raw material was achieved by spray-drying. MCC is mainly used as dry binder in manufacturing of tablets as well as in wet granulation, and it is the most important excipient in the wet extrusion process [35].

The interaction of cellulose and drug components at the structural level is not excluded, and this may appreciably affect the properties of solid medicinal forms: strength, rate of disintegration in various media, etc [39]. The adsorption of various drugs on microcrystalline cellulose (MCC) suspended in aqueous solutions, which will alter the dissolution time [38]. It was found that MCC requires more water for pellet formulation [37].

It was found that the difference in MCC particle size resulted in varying amounts of water needed for the process [40] and this will be disadvantageous for highly water soluble drugs like L-arginine free base and its salt HCL to be extruded by using MCC as an extrusion/spheronization aid.

Hydrophilic matrices draw attention in the search for improved patient compliance and decreased incidence of adverse drug reactions. Under ideal conditions, a sustained- release formulation maintains therapeutic blood level of a drug for a specific period of time. Oral controlled-release dosage forms have been developed and studied to restrict these systems to specific regions of the gastrointestinal tract as well as to improve the pharmacological activity and to reduce toxic effects. One method of fabricating controlled-release formulations are by the incorporation of the drug in a matrix containing a hydrophilic, rate-controlling polymer. Hydroxypropylmethylcellulose (HPMC) is the dominant hydrophilic vehicle used for preparation of oral controlled drug delivery systems [42] and the polymer most widely used as the gel-forming agent in the formulation of solid, liquid, semisolid and even controlled-release dosage forms. Water penetration, polymer swelling, drug dissolution, drug diffusion and matrix erosion from these dosage forms are controlled by the hydration of HPMC, which forms a gel barrier through which the drug diffuses. The adjustment of the polymer concentration, the viscosity grade and the addition of different types and levels of excipients to the HPMC matrix can modify the drug release rate [43]. HPMC has been used as an alternative extrusion/spheronization aid to produce pellets without the incorporation of MCC [44] and used to produce sustained-release tablet of L-arginine [45].
The aim of the present investigations was to obtain pellets by using powdered MCC or HPMC as a binder and extrusion/spheronization aid with L-arginine free base and its salt HCL according to the reasons mentioned above, 4 different formulations (table 23) were formulated and fed from a gravimetric dosing device into the barrel of a twin-screw extruder.

Table 23: Formulations of L-arginine free base and its salt HCL for wet extrusion.

Component	FA % w/w	FB % w/w	FC % w/w	FD % w/w
L-arginine free base	60	60	-	-
L-arginine HCL	-	-	60	60
MCC	40	-	40	-
HPMC 50 cP	-	30	-	30
HPMC 4000 cP	-	10	-	10

It was not successful to obtain extrudates because L-arginine free base and its salt HCL was very water soluble and for this reason we received a sticky mass instead of uniform extrudates.

2.3 Solid lipid extrusion

Lipids are a broad group of naturally-occurring molecules which includes fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E and K), monoglycerides, diglycerides, phospholipids, and others [32]. Although the term lipid is sometimes used as a synonym for fats, fats are a subgroup of lipids called triglycerides. Lipids also encompass molecules such as fatty acids and their derivatives (including tri-, di-, and monoglycerides and phospholipids), as well as other sterol-containing metabolites such as cholesterol [36]. Solid lipids are important excipients in the pharmaceutical technology due to their properties, which are non-toxic, biodegradable products and not expensive. They are used as lipid matrices with a variety of different functions, which are sustained release of many drugs, improvement the dissolution rate and bioavailability of poorly water-soluble drug can be significantly enhanced by the preparation of solid dispersions using lipid carriers by spray drying technique [46, 55, 56, 62, 68, 70, 71], suppressing and taste masking of bitter tasting drugs [48-51], and floating drug delivery systems [52-54, 70].

These systems are useful to several problems encountered during the development of a pharmaceutical dosage form and for decreasing the side effect of some drugs that have gastric irritation [66].

There are studies used solid lipids for sustained release matrices [57-62, 70]. The physical properties instability of the lipids during their storage is the major disadvantage of using them in pharmaceutical formulations. The aging of the lipid may lead to a change in melting ranges, melting enthalpy, producing pores on the surface, rheological properties and in tensile strength [60, 63, 64]

The sustained release dosage forms sometimes contain large doses of the active ingredients and the physical properties of lipids during their storage are very important aspect for sustained release dosage forms. So, during the storage and aging of lipids their physical properties may be affected. Some studies have been shown a change in drug release properties from lipid matrices after storage [63, 65-67]. For example, release profiles of salbutamol sulfate from the capsules elaborated were dependent on the type of Gelucires®, fast release, in the case of Gelucire® 35:10, a slower release for Gelucire® 48:09 and a slow release for Gelucire® 46:07, which are stored at room temperature for one year [69].

The effects of storage on the dissolution profiles of ambiently cooled systems were studied by Sutananta et al. [72] with increase theophylline release from Gelucire® 55/18 and 50/13 matrices on ageing for up to 180 days. In vitro differences in Ketoprofen dissolution profiles between freshly manufactured and stored capsules from mixtures of Gelucire 50/13 and 50/02, were shown not to be significant in vivo [73].

The most common used preparation methods for lipids are production of tablets by melting or compression [76], melt granulation [77, 78], liquid filling of hard gelatine capsules, hot-melt extrusion [31, 74], cold extrusion [48, 50, 51] and spray-congealing using ultrasound [75].

One of the most often used preparation method for lipids involve hot melting above their melting ranges and then solidification of the melted lipids by using different methods for that. On the other hand, it was found that lipids can be extruded at temperatures below their melting ranges [33, 48, 50, 51]. It is shown that physical techniques using X- ray diffraction, differential scanning calorimetry DSC and Raman spectroscopy measurements have enabled to relate the molecular-level structures and crystallization kinetics of fats and lipids. The influences of generation of different lipid polymorphic forms and on crystallization kinetics are dependent on crystal seeding and shear stresses [80].

In this work the solid lipid extrusion/spheronization was used to obtain physically stable sustained release spherical pellets. The extrusion/spheronization process was done at moderate temperatures, in which lipids were treated below their melting ranges.

2.3.1 Formulation variation

Two commercially available powdered lipids were chosen as binders for lipid pellets formulation and extruded below their melting ranges. They have different composition, but similar melting range: glyceryl palmitostearate (Precirol® ATO5) and glyceryl trimyristate (Dynasan 114®) were chosen to obtain sustained release spherical pellets and to compare between them. They possess different structures and constituents which allow the generation of broad information about the dependency between the observed results and the lipid composition. Four different formulations were prepared (table 24), the mixtures of lipids with L-arginine free base and it is salt HCL were extruded at different extrusion temperatures to see thermal effect on the physical properties and release behaviour of the obtained pellets. Drug release and melting enthalpy of the obtained pellets were measured in order to analyze their stability during storage influenced by time, structure and storage conditions.

Table 24: Composition of formulations based on lipids as binders (numbers indicate the percent fraction of the lipid in the formulation).

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Component	A % w/w	B % w/w	C % w/w	D % w/w
L-arginine free base	50	50	-	-
L-arginine HCL	-	-	50	50
Precirol® ATO5	50	-	50	-
Dynasan® 114	-	50	-	50

2.3.2 Process parameters

The process parameters used for all formulations were the same. Only cylinder temperatures for all formulations were adjusted differently. The powder feed rate was always constant 40 g/min. The mass was extruded through a die plate with 23 holes of 1 mm diameter and 2.5 mm length. They were extruded at a constant screw speed of 30 rpm. Material temperature was measured next to the die plate just before the extrusion step.

In order to analyze the effect of using different cylinder temperatures to obtain sustained release spherical pellets and to compare between them, cylinder temperatures were modified. Utilizable lipid extrudates should be obtained. Therefore material temperatures had to be high enough to allow the lipid/drug mass to be extruded uniformly through every hole of the die plate which was not possible at too low temperatures. Otherwise the lipid/drug mass had to be rigid enough to give compact extrudates; after extrusion through the die plate deliquescence and curling of the extrudate must not occur which were the effects of too high material temperatures.

Powder mixtures of pure Dynasan® 114 or Precirol® ATO 5 with L-arginine free base and it is salt HCL could be processed in a twin screw extruder at different cylinder temperatures below their melting ranges (52-56°C).

An equilibration of the material temperature at the die plate was reached after several minutes of extrusion.

The powdered lipids were mixed with 50% of L-arginine free base and it is salt HCL powder. Again, the material temperature was measured in dependence of the adjusted cylinder temperature. Drug amounts of 50% of L-arginine free base and it is salt HCL could be processed without any restrictions, the extrusion process was found to be robust. Under the process conditions the extrusion aids were not thoroughly melted. At lower cylinder temperatures of 46-50°C, all of the tested formulations showed problems during extrusion and the mass could not be extruded homogeneously through the die holes and when the die holes were blocked, material temperatures at the die plate exceeded the cylinder temperature. This increase in temperature was supposed to be the result of friction forces between the particles, as L-arginine is insoluble in the lipid base and remained solid during extrusion. At higher cylinder temperatures above 50°C the material temperature remained below the adjusted temperature again and only A and B of the tested formulations showed no problems during extrusion. Suitable smooth extrudates were produced, see table 25. This means that it was impossible to get suitable rigid, smooth extrudates from the C and D formulation. Friction effects only seemed to be relevant at low cylinder temperatures.

Dynasan® 114 contains more than 90% glyceryl trimyristate. The melt of glyceryl trimyristate solidifies in a way becoming white and clouded. This change of color is the effect of recrystallization going along with an increase in volume and porosity and this effect was seen in the work of Whittam et al. [88] and Reitz et al. [33].

The different melting points of the chosen lipids are shown in table 37, and typical DSC curves with the melting peaks of the powdered lipids are displayed in figure 15. Hence, binding of the drug substance is achieved by softening and melting of the lipid binders.

Table 25: Five batches of A and B formulations extruded at different cylinder temperatures.

Formulation	Material temperature	Cylindrical temperature	rpm
A1	50°C	56°C	30
A2	49°C	55°C	30
A3	48.5°C	53°C	30
A4	48°C	52°C	30
B1	50°C	52°C	30

Optical evaluation of the extrudates of A and B formulations led to the conclusion of having the best extrusion conditions at material temperatures between 48 and 50°C for the 50% lipid/drug loaded mixtures.

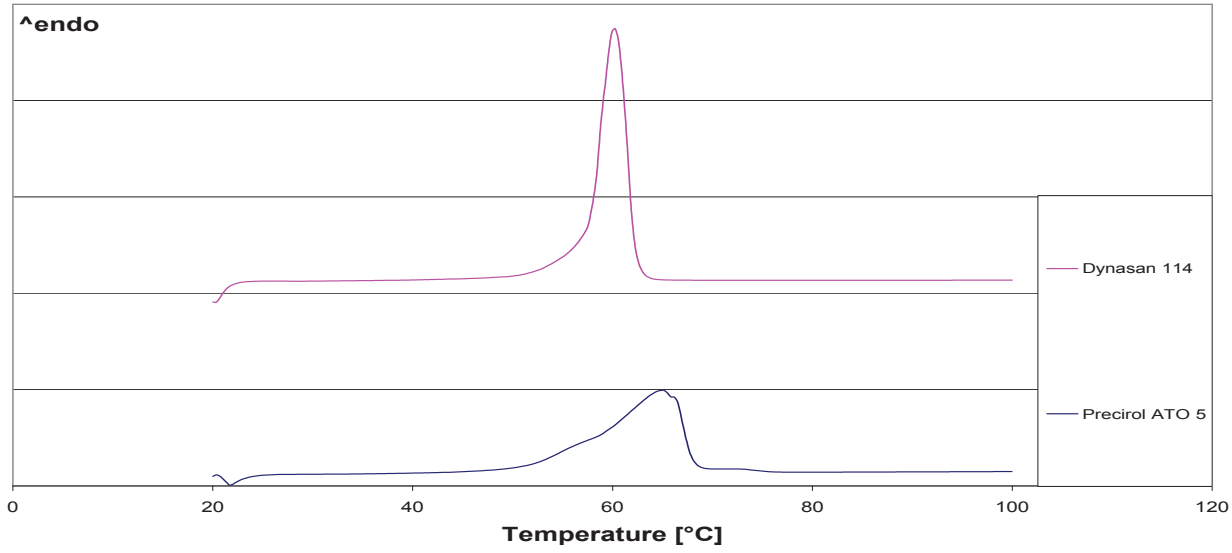

Figure 15: DSC tracks of untreated lipid powders, recorded at a heating rate of 10 K/min. All signals are scaled to sample weight.

2.3.3 Spheronization

The rounding of the extrudates in a spheronizer to give round pellets with narrow particle size distribution offers some advantages. The products are characterized by a reproducible and defined shape and surface and they are suitable for filling into hard gelatine capsules, stick-packs and sachets. However, the spheronization process requires special mechanical properties of the extrudates. The mass should dispose of certain brittleness, so that the extrudates brake into short segments. On the other hand the mass should dispose of certain plasticity, which is required for the spheronization to round pellets.

High melting lipids can show the above mentioned mechanical properties in dependency of the temperature. They are brittle at room temperature and soften within a broad temperature range due to their heterogeneous melting properties, in which they are plastically deformable without sticking. Thus, spheronization can be performed in a tempered spheronizer with rising extrudate temperatures [81].

Spheronization process is a manufacturing tool to get suitable sustained release matrix spheres. The solvent-free spheronization of lipid extrudates in a spheronizer has been described [50, 51, 81]. Spheronization of lipid extrudates in a spheronizer requires suitable process and formulation parameters that allow a stable and robust manufacture of pellets with reproducible product properties. Spheronization experiments on the lipid extrudates were performed, showing the influence of formulation and process parameters on the spheronization quality of the extrudates. Rounding temperature is decisive for the spheronization of the lipid extrudates. The obtained pellets are analyzed with respect to their suitability as sustained release matrix spheres in drug dissolution studies.
The spheronization process will be the critical step to get spherical pellets form by using solid lipids. The five batches of A and B formulations that had been extruded at different cylinder temperature were rounded after extrusion at different spheronizer speeds, temperatures and at different time intervals (table 26).

300 g of every batch were spheronized for up to 11 min. The five batches of A and B formulations were characterised by image analysis.

Table 26: Five batches of A and B formulations were spheronized at different spheronization conditions.

Formulation	Temperature	rpm	Time (min)	Aspect Ratio
A1	50°C	1500	6	1.05
A2	40°C	1500	11	1.08
A3	50°C	1500	6	1.07
A4	50°C	1000	6	1.88
B1	50°C	1500	6	1.63

Seven shape parameters are suggested to characterise the shape of pellets, but roundness and elongation most effectively distinguished pellets from each other [82]. The aspect ratio (a length-width ratio) could well be used for extrusion/spheronization; the aspect ratio sufficiently discriminates between the different pellets, in spite of the differences in shape. Therefore, the aspect ratio is considered as a suitable method for determining the shape of these types of pellets [83, 85, 86]. The suitable aspect ratio mean was equal to or lower than 1.2 regarded as sufficient for pharmaceutical pellets, but if the aspect ratios means above 1.2 were considered as insufficient for pharmaceutical pellets and give an indication of more or less cylindrical granules [84].

The rounding properties of A1 and B1 batches extrudates were different in spite of using the same spheronization conditions (table 26); this effect may be due to lipid binder used, Precirol® ATO 5 used in A1 batch and Dynasan® 114 in B1 batch. This mean Precirol ATO 5® have better rounding properties than Dynasan® 114 during spheronization. Therefore, the batch A1 was easier to be spheronized and to get suitable spherical pellets than the batch B1 under the same selected spheronization conditions.

The fine powder fraction as well as electrostatic interactions, size and shape of the rounded particles resulted from friction heat in the spheronizer, which is caused by interparticular friction between the rotating pellets and the friction between pellets and the spheronizer itself. The resulted powder fraction from B1 batch was more than A1 batch and this due to the same mentioned reasons above.

It was interesting to observe that the higher the temperature used the more efficient was spheronization, but the temperature used far below the melting point of the solid lipid as well as the temperature very close to it, it was insufficient and not effective for rounding of the pellets. Sticking of the pellets at the wall and with each other was observed by using very high temperature; speed of the spheronizer and for long processing time due to melting of the lipid binder and only fragmentations of extrudate were obtained by using low temperature; speed of the spheronizer and for short processing time due to insufficient energy introduction and the temperature was not high enough to allow softening of the extrudates.

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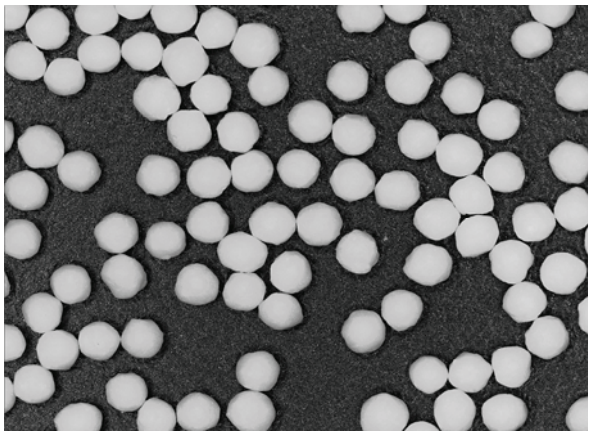
Rounding of the pellets was influenced by the temperature and speed of the spheronizer, as high as possible temperature and speed of the spheronizer used, result in an effective rounding of the pellets and this lead to decrease processing time influenced rounding of the pellets and decrease of the aspect ratio occurred relatively rapidly. Table 26 shows aspect ratio of the pellets as a function of the temperature and speed of the spheronizer. These results illustrate that processing time for an efficient spheronization could be shortened by rising the temperature and speed of the spheronizer. Thus, choice of the 50°C temperature and 1500 rpm speed of the spheronizer and processing time for 6 min was decisive for efficiency and robustness of the rounding process and allowed for the spheronization of lipid extrudates to give round pellets with an aspect ratio of < 1.05.

2.3.4 Pellet shape, size and size distribution

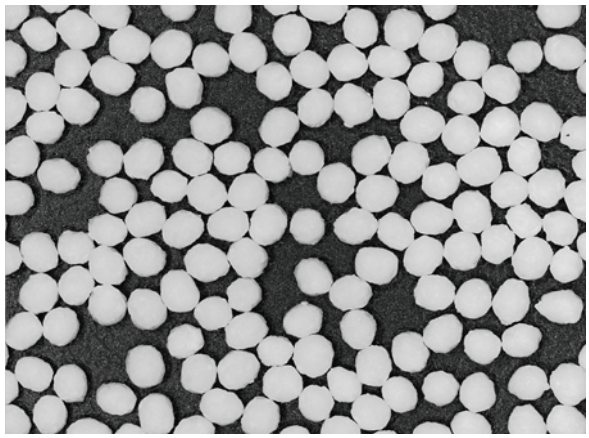
The morphological characteristics of pellets are critical parameters, because the physico-chemical features depend on the size, shape and surface geometric of the particles. To ensure the spherical shape and required particle size is a prerequisite. The detailed technology is basic requirement for the successful and cost efficient production of particles of acceptable quality. Since the determination of the particle size is influenced by the particle shape, microscopic examination is suggested, which together with image analysis is suitable for the assessment of the most typical parameters. The method of the microscopic image analysis is useful not only for particle size measurement, but also for particle shape and texture evaluation, with a high sensitivity. Using the microscopic method particle shape may be defined either qualitatively and/or quantitatively [87]. Pellet imaging was performed on each batch of pellets under the same optical conditions (figure 16). The particle size distribution of spherical pellets was determined using a set of test sieves (0.8-2 mm).

The equivalent diameter for lipid pellets ranges from 1.52 to 1.79 mm, depending on the lipid used as in A1 and B1 batches, but in A1-A4 batches, it strongly depended on the used temperature and speed of the spheronization process, together with the processing time.

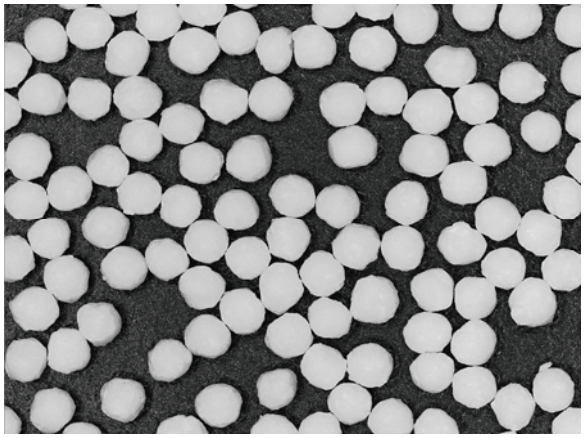
The yield percentage (0.8-2 mm) was very good and high for all batches. The batches A1-A3 had a 10% interval higher than 90% and their aspect ratios ranged from 1.05 to 1.08 respectively, which is very lower than other batches. The formulation of A4 and B1 10% intervals were above 50% and their aspect ratios were 1.63 and 1.88 respectively (table 27 and figure 17). Therefore, using Precirol®ATO 5 as a lipid binder to produce pellets regarding their size distribution and shape was better than using Dynasan® 114, in spite of having similar melting points. This may be due to their different chemical composition, structure and different particle sizes. Precirol® ATO 5 has smaller particle size than Dynasan® 114.



A1

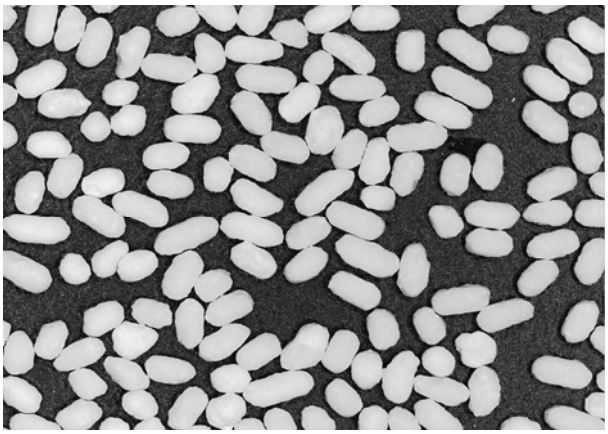


A2



A3

A4



B1

Figure 16: Image analysis of the five batches of A and B formulations (Pictures of the spot samples gave first hints on the rounding process).

Table 27: Pellet's morphological properties of the five batches of A and B formulations.

Formulation	Equivalent dia. (mm)	Yield 0.8 - 2.0 mm (%)	10% - interval (%)	Aspect Ratio
A1	1.68±0.07	98.48	95	1.05
A2	1.55±0.14	98.42	90	1.08
A3	1.79±0.06	99.10	100	1.07
A4	1.68±0.21	97.46	54	1.88
B1	1.52±0.15	97.38	62	1.63

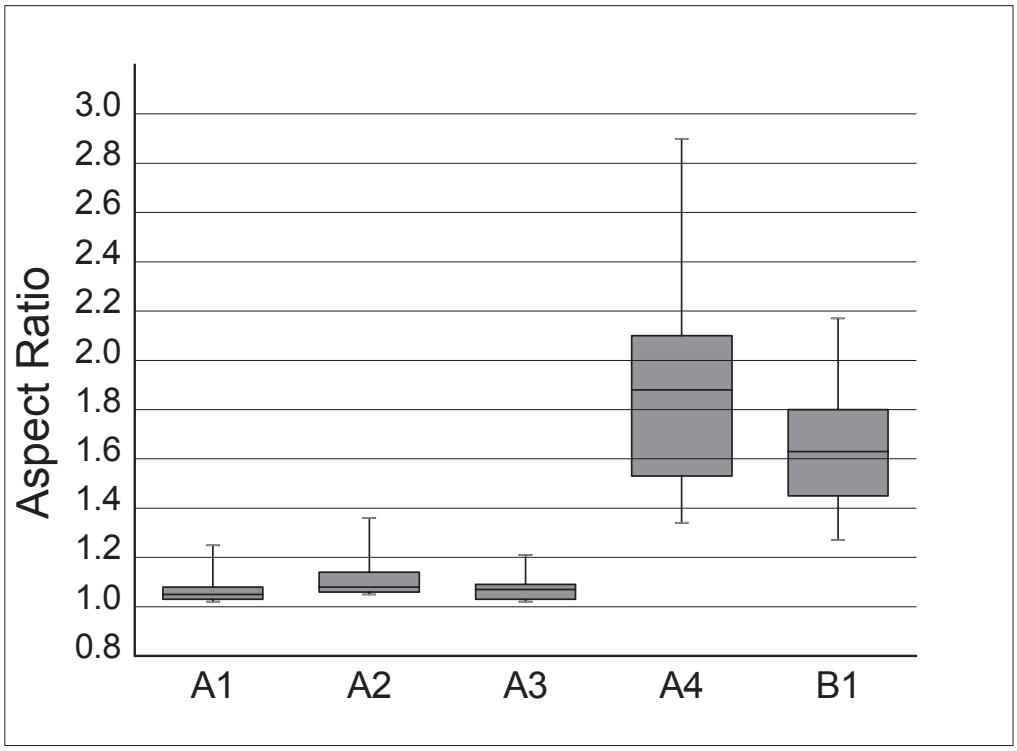


Figure 17: Aspect ratios of pellets of the five batches of A and B formulations (x1, x25, x50, x75, x99, n>500) with different lipid binders made by solvent-free solid lipid extrusion/spheronization process.

2.4 L-arginine free base dissolution

2.4.1 Dissolution media and process parameter

To investigate the influence of lipid binder type and extrusion/spheronization conditions on the dissolution behaviour of the obtained pellets, dissolution tests were performed for all batches. In addition, the effects of storage conditions on the dissolution behaviour of the obtained pellets of all batches were investigated. In order to ensure complete wetting of the pellets and to prevent them from floating during dissolution test, the basket method was used. The rotating speed of the baskets was kept constant at 75 rpm during dissolution tests of all batches, because the obtained pellets were designed for sustained-release purpose.

The results of in vitro dissolution testing for the five batches of A and B formulations show that all the batches released about 80% of the L-arginine content within the first 40 min, A4 and B1 have released about 100% of the L-arginine content at 90 min (figure 18). The focus of our study was to obtain sustained-release profiles and not intermediate-release profiles. It was difficult to get sustained released formulations by using lipid matrices in spite of their low porosity, which was approximately 6% and using different extrusion and spheronizing conditions. This might be due to very high water solubility of L-arginine that is rapidly released from the lipid matrices when come into contact with the dissolution medium. The findings are in sharp contrast to the work of Michalk et al. [48] and Reitz et al. [33] on solid lipid extrusion of poorly soluble drug substance.

A potential explanation to understand the mechanism behind the observed dissolution effects, especially the small difference in the dissolution profiles can be formulated as follows. During the manufacture of extrudates, the suspended drug particles evade the resistance of the die plate walls while in motion and a very thin layer mainly composed of softened lipid is formed at the smooth die plate wall. This phenomenon is known as the ‘slip’ or ‘wall depletion’ effect [90] and has been observed during extrusion of lipids with high disperse-phase loading levels. As a result a smooth pellet surface is obtained by spheronization process with the surface composed mainly of lipid [89].

Dissolution of L-arginine from the lipid pellets is controlled by diffusion because lipid matrix stays intact after the release of the L-arginine and is therefore strongly influenced by the matrix formulation, according to the square root kinetics of Higuchi. Higher drug solubility leads to faster drug release and L-arginine regarded as highly water soluble drug. Release of an embedded drug from an inert porous matrix can be explained by diffusion processes in a simplified way. The rate of the diffusion steps is mainly controlled by the concentration gradient that is highest at the matrix surface at sink conditions and lowest within the matrix due to hindered exchange with the release medium because of fine pore capillaries. Hence, drug particles with direct contact to the release medium are dissolved more quickly due to the high concentration gradient.

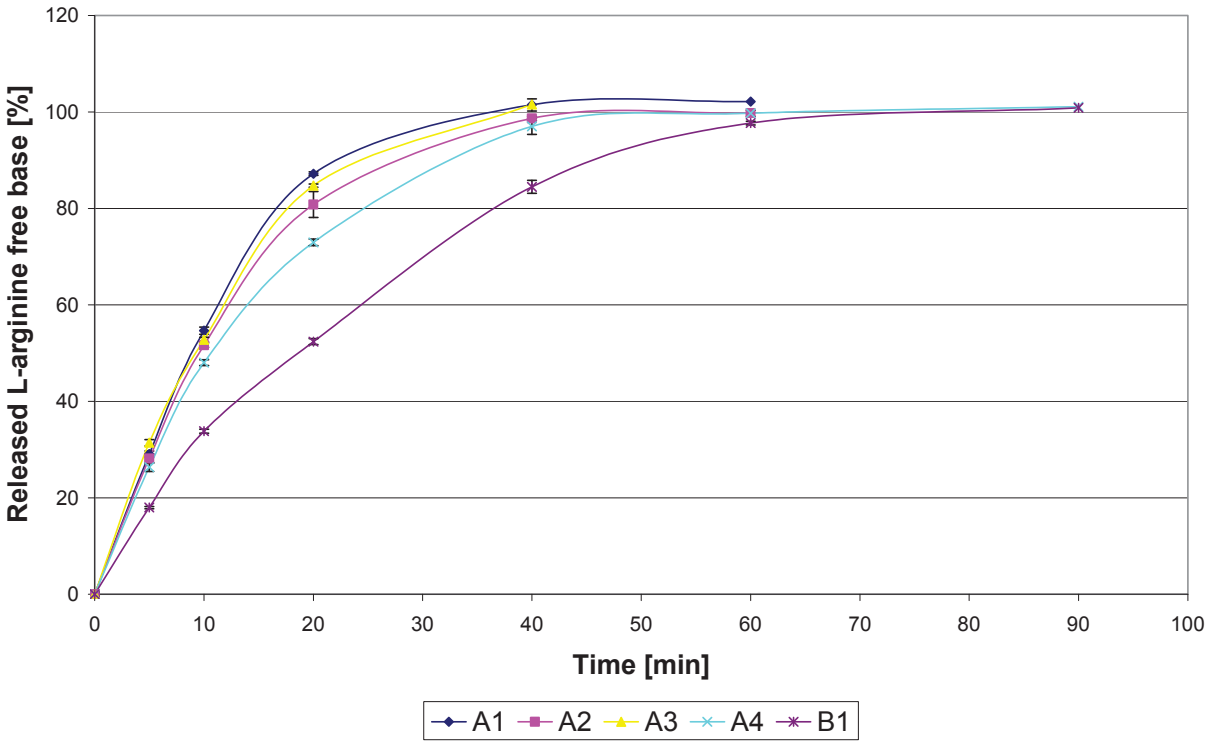


Figure 18: L-arginine free base release from lipid pellet formulations. Dissolution media: Phosphate buffer (mean ± SD, n=6), temperature: 37± 0.5 °C, 75 rpm, basket method.

Fast release of surface drug particles produces a burst effect. Particles without direct contact to the surface of the matrix are dissolved more slowly and are released into the medium after an additional diffusion passage through the matrix [89].

Our aim was to obtain sustained-release pellets by using solid lipid extrusion/ spheronization process, but the results of in vitro dissolution test for all batches, by using dissolution medium (phosphate buffer at a pH of 6.8) show that pellets have intermediate-release instead of sustained-release properties.

To see the influence of using different dissolution media at 37°C with different pH and ionic strength on the dissolution behaviour of the obtained pellets, dissolution tests were done for all batches. 0.1 N HCL solution with a pH of 1 and purified water with a pH of 7 were used as dissolution media.

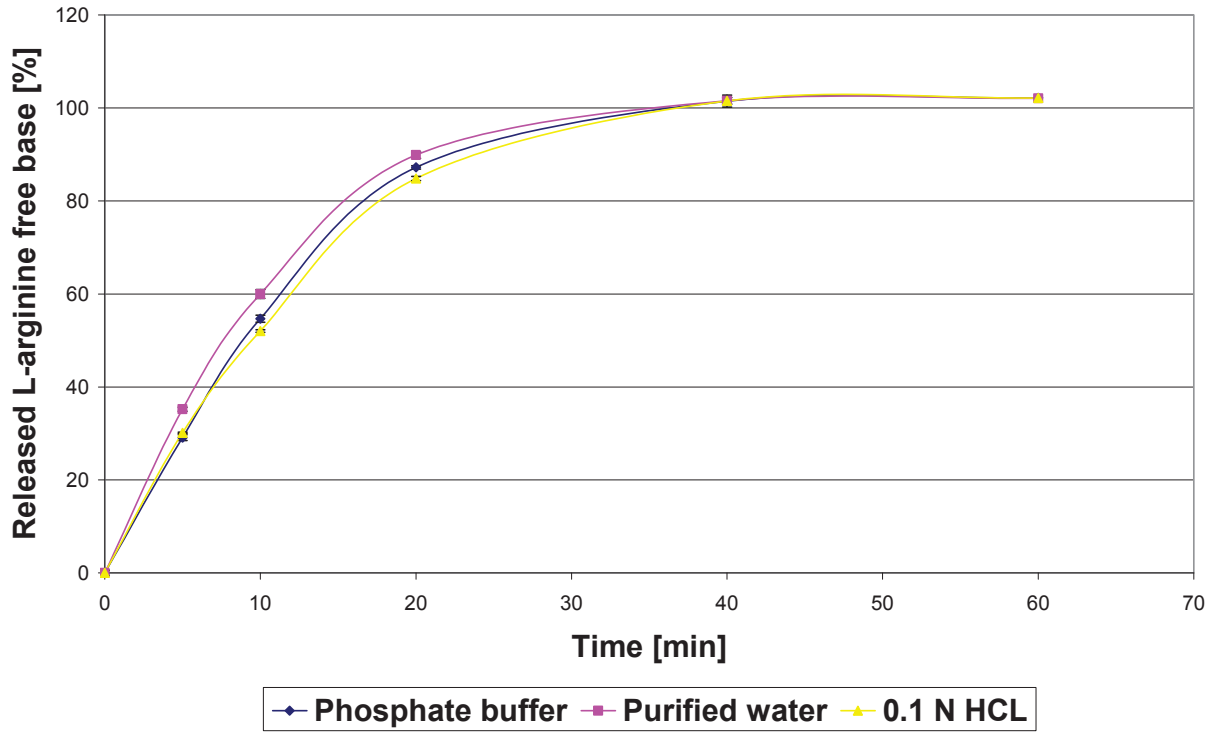


Figure 19: Influence of dissolution media type on release characteristics of A1 batch pellets (mean ± SD, n = 6). Temperature: 37± 0.5 °C, 75 rpm, basket method.

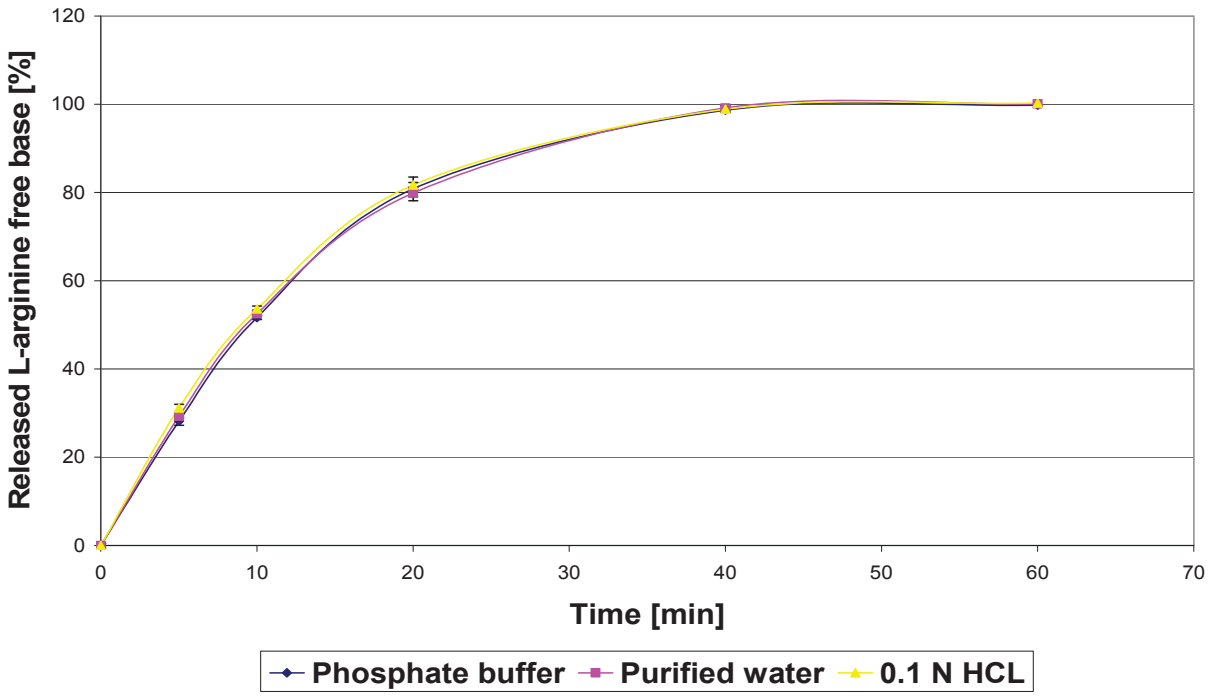


Figure 20: Influence of dissolution media type on release characteristics of A2 batch pellets (mean ± SD, n = 6). Temperature: 37± 0.5 °C, 75 rpm, basket method.

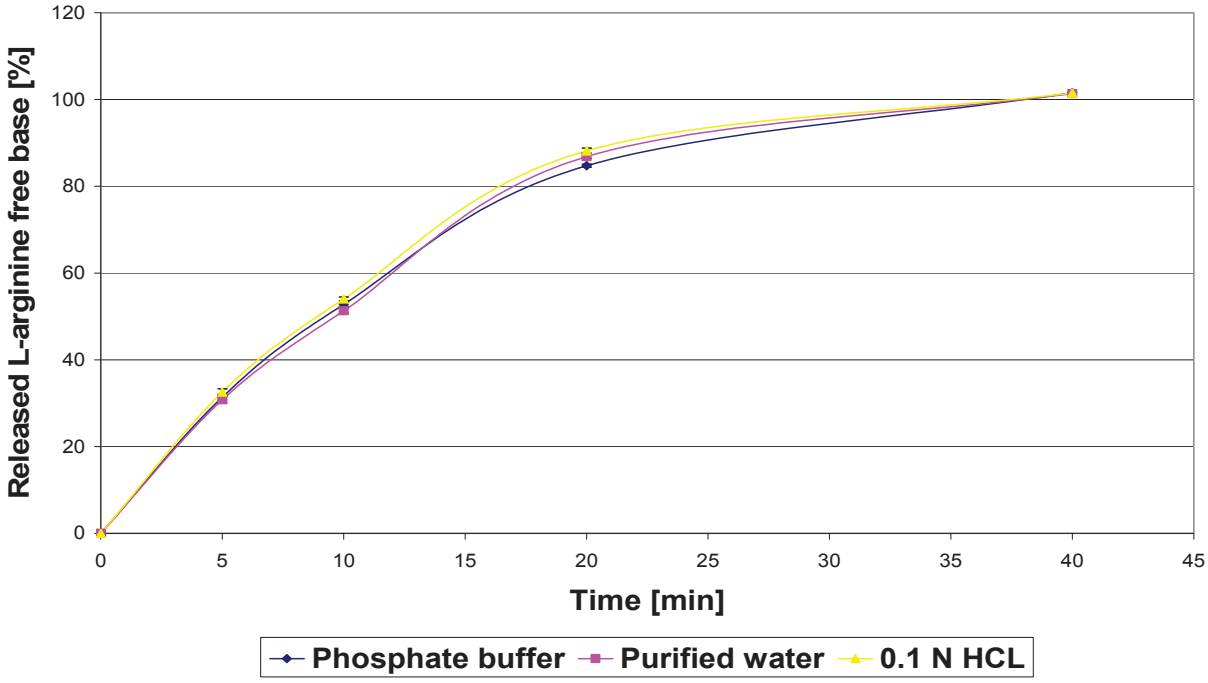


Figure 21: Influence of dissolution media type on release characteristics of A3 batch pellets (mean ± SD, n = 6). Temperature: 37± 0.5 °C, 75 rpm, basket method.

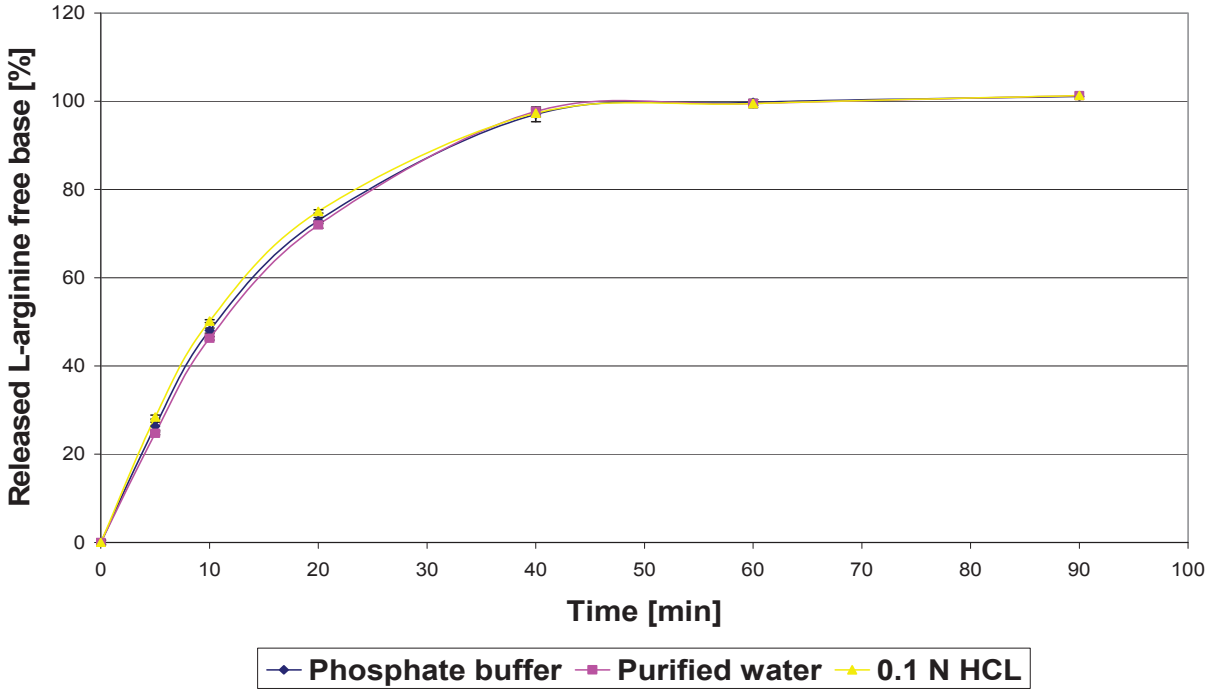


Figure 22: Influence of dissolution media type on release characteristics of A4 batch pellets (mean ± SD, n = 6). Temperature: 37± 0.5 °C, 75 rpm, basket method.

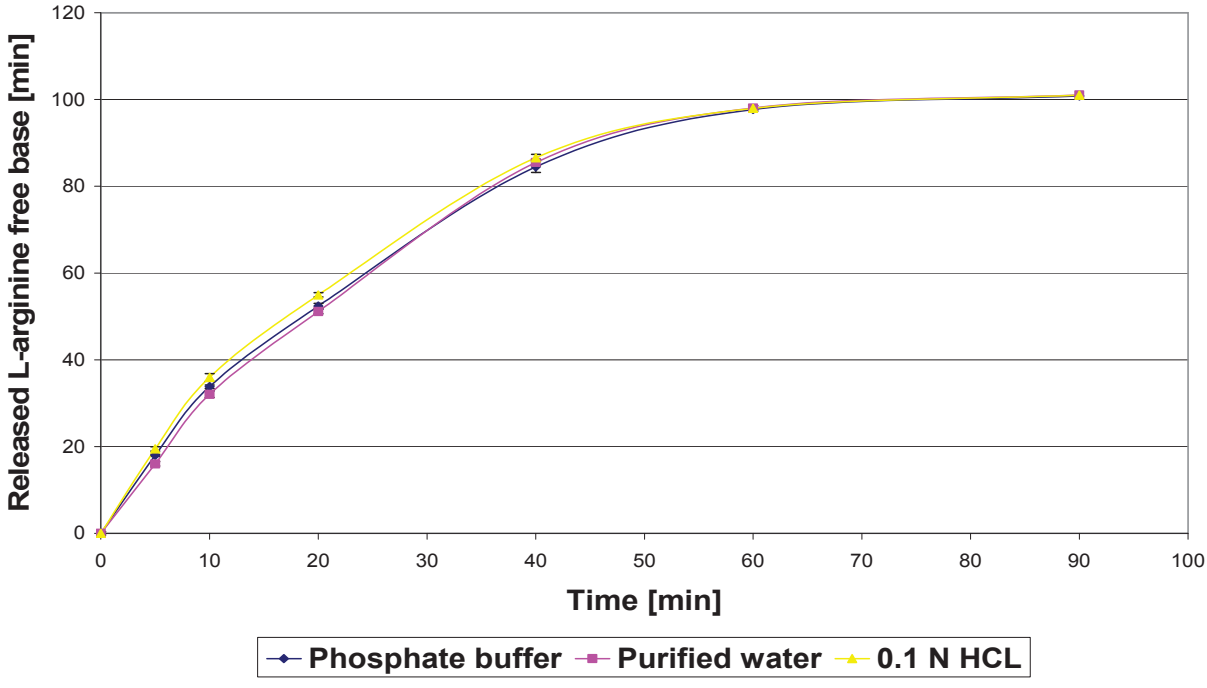


Figure 23: Influence of dissolution media type on release characteristics of B1 batch pellets (mean ± SD, n = 6). Temperature: 37± 0.5 °C, 75 rpm, basket method.

As displayed in figures 19-23, no significant differences between the drug releases into different dissolution media could be detected at 37°C (p-value > 0.05). This applied to all pellet batches regardless of lipid binder and extrusion/spheronization conditions used.

2.4.2 Self-coating of the lipid pellets

During spheronization this may lead to a plasticization of lipid binder in the outer parts of the pellets, regardless their melting points and initial composition. DSC measurements can be used to prove the melting behaviour of samples and therefore give information about their physical properties.
The melting point of all pellet lipid batches containing Precirol® ATO 5 (A1-A4) and that contain Dynasan® 114 (B1) display the same peaks or shoulders in the DSC measurements after the first and second heating of the samples, as can be seen in figures 24 and 25, irrespective of their dependent on the extrusion/spheronization temperature and conditions used. No differences could be determined. Therefore there is no indication that there are measurable differences in the chemical composition of the samples.
A possible mechanism to explain the observed effects is the assumption of a lipid coating of the pellets. During the extrusion process, although heat is used to plasticize the mixtures of lipid binder and L-arginine free base before the die passage, due to friction, heat and pressure forces, on the extrudate surfaces in the extruder and/or in the die plate the temperature at the outer surface of the extrudate might be higher.

This can result in a partial melting of the lipid components and changes in the morphology due to recrystallization. Consequently, the exposure of incorporated drug particles might be different on the outer extrudate surface and new surfaces generated.

In addition to spheronization, extrudates involve a complete thermo-mechanical treatment of the extruded surfaces by external heat and frictional heat of the pellet bed, which may lead to partial melting of the lipid binder and formation of spherical pellets.

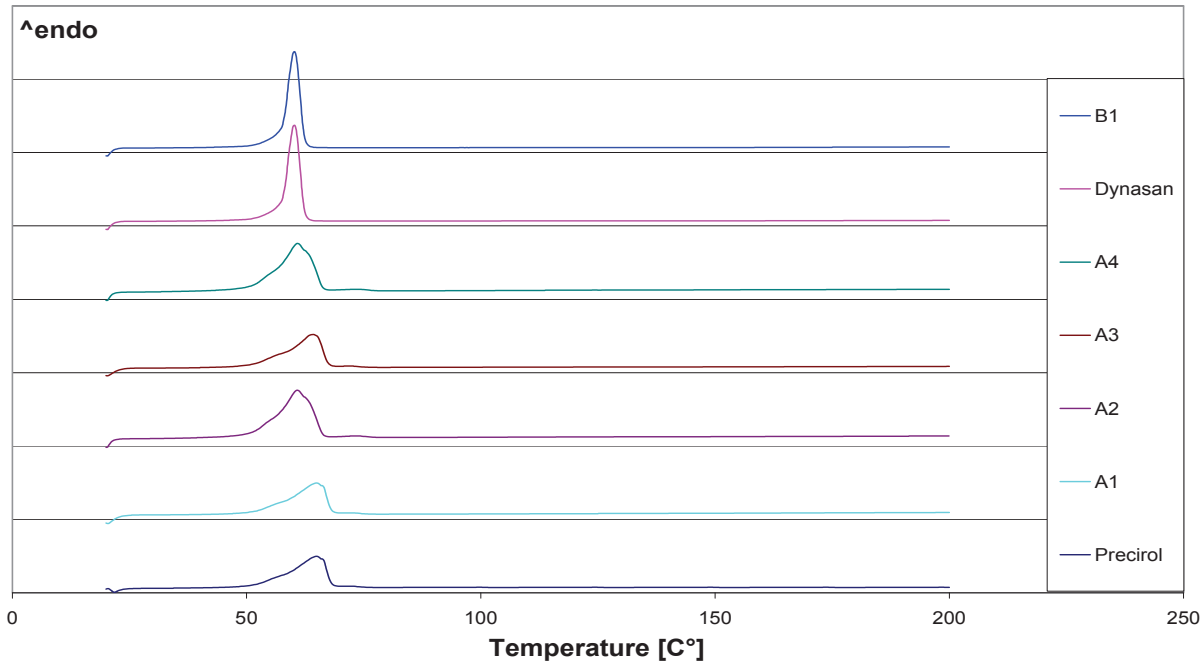


Figure 24: DSC tracks of different lipid pellets, recorded at a heating rate of 10 K/min, first heating. All signals are scaled to sample weight.

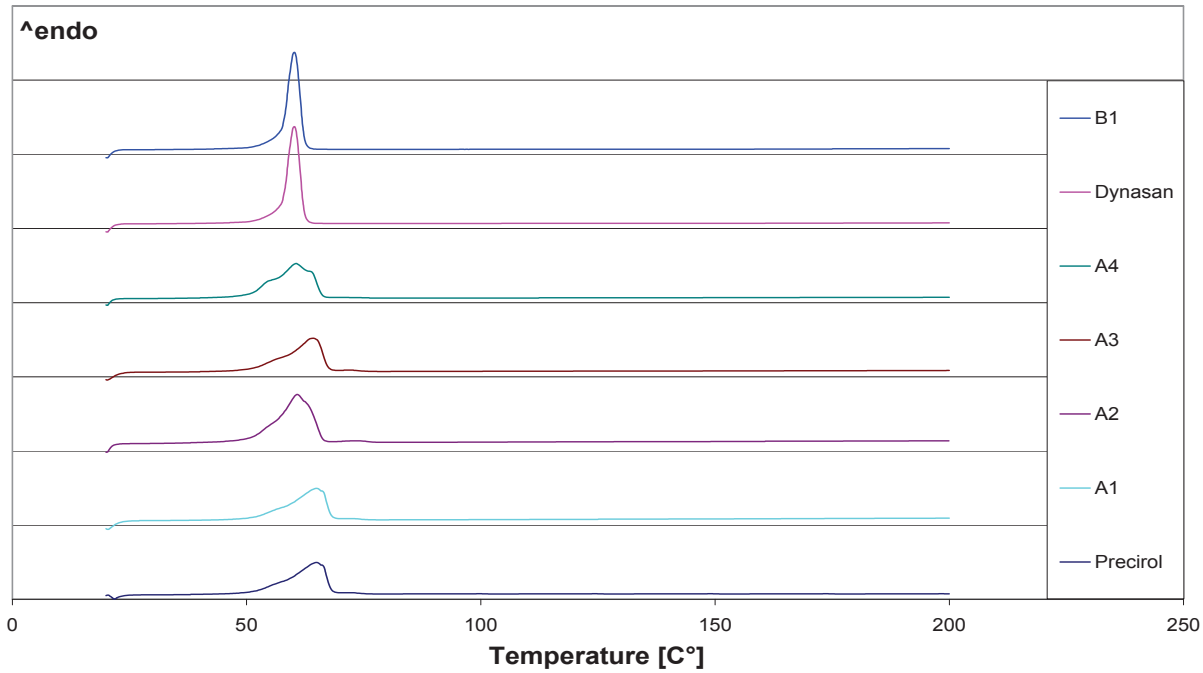


Figure 25: DSC tracks of different lipid pellets, recorded at a heating rate of 10 K/min, second heating. All signals are scaled to sample weight.

The molten parts of the lipid binder layer will separate during spheronization process and concentrate on the surfaces of the pellets and this lead to coating of each pellet by a layer of molten lipid. The composition of this coat will determine the release behaviour of the pellets. The lipid binder and active crystalline drug will be distributed in a minor amount in this coat and at high percentage in the core of the pellets.

The scanning electron micrographs of cross-sections of the pellets were taken and capable of displaying the mechanism of self-coating through the lipid binder. The term self-coating has been recently introduced [91].

In figure 26, the cross-sections of pellets batches A1 and A2 are displayed. The black arrows point to the coating layer around the pellet which is detectable even at small magnification (100x, as adjusted at apparatus) and at large magnification (1000x, as adjusted at apparatus). The coating layer around each pellet is clearly visible and an approximately 10 μm thickness layer around the core of the pellets which acts as a diffusion barrier.
The hypothesis that the lipid binder and active crystalline drug will be distributed in a minor amount in the outer coating layer and a high percentage in the core of the pellets is supported by the scanning electron micrographs obtained for all pellet batches, as seen in the figures 26, 27, 28.

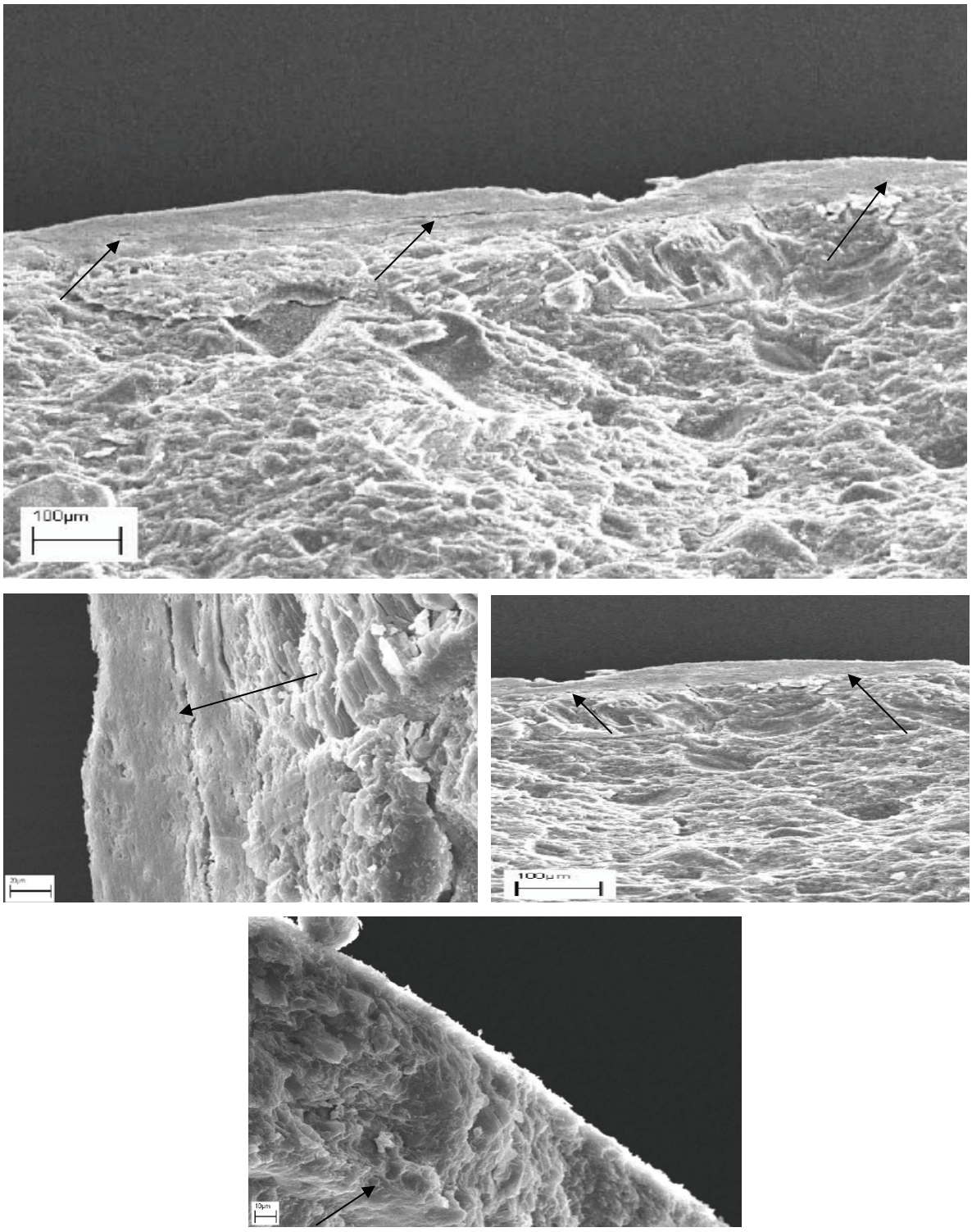


Figure 26: Scanning electron micrographs of pellets containing 50% L-arginine free base, 50% Precirol® ATO 5 (batches A1 and A2), taken at an adjusted apparatus magnification of 100 and at an adjusted apparatus magnification of 1000.

In general the outer layers of all pellet batches appear relatively smooth and congealed and beneath the outer layer a rather rough and almost needle-like structure can be seen.

In figure 27 the same pellet structures are displayed at larger magnifications. Again a distinct layer of approximately 10 µm can be distinguished on the surfaces of the pellet and beneath a thicker layer of different texture.

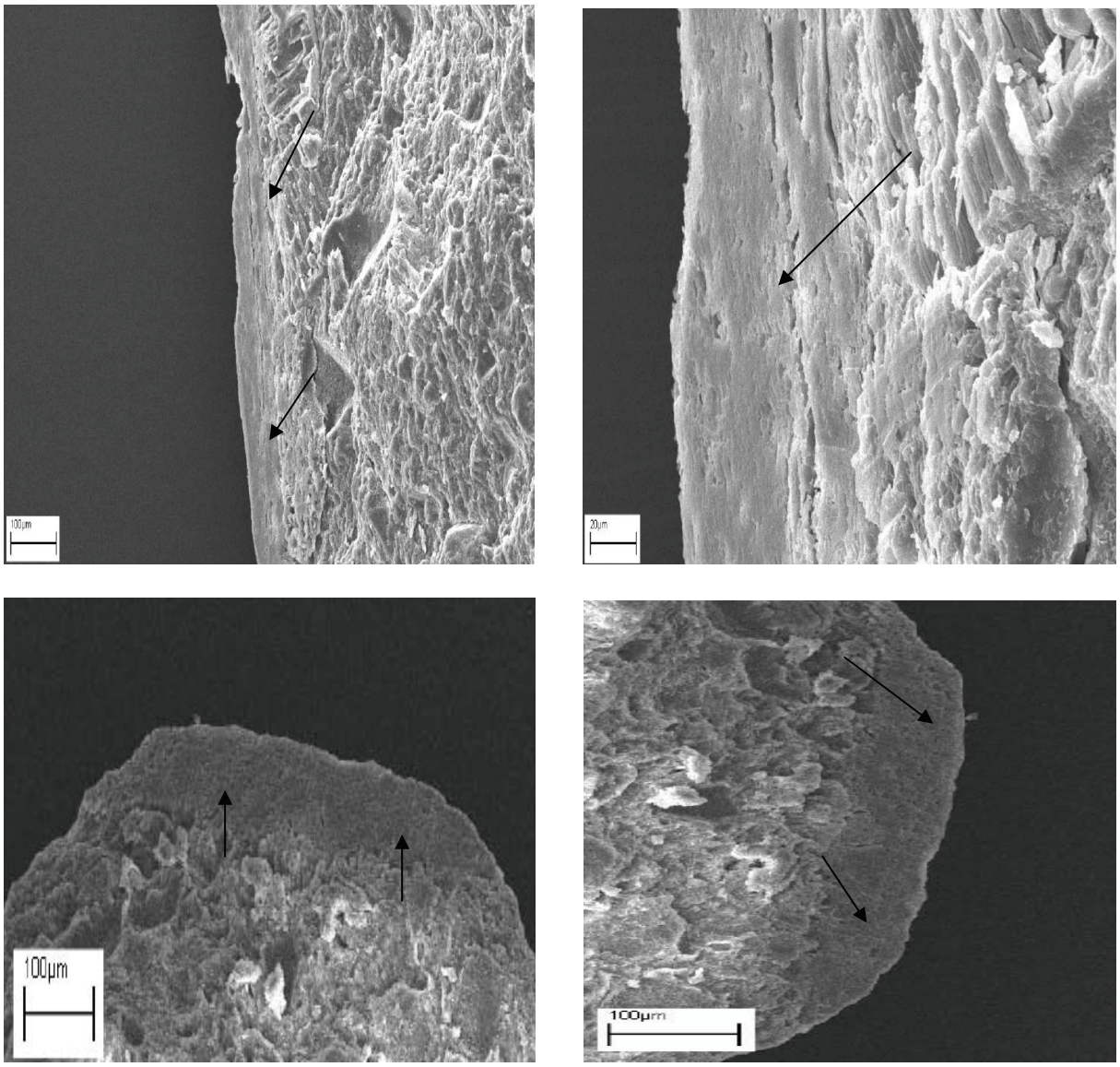


Figure 27: Scanning electron micrographs of a pellet batches (A3 and A4) section with lipid layer and subjacent structures.

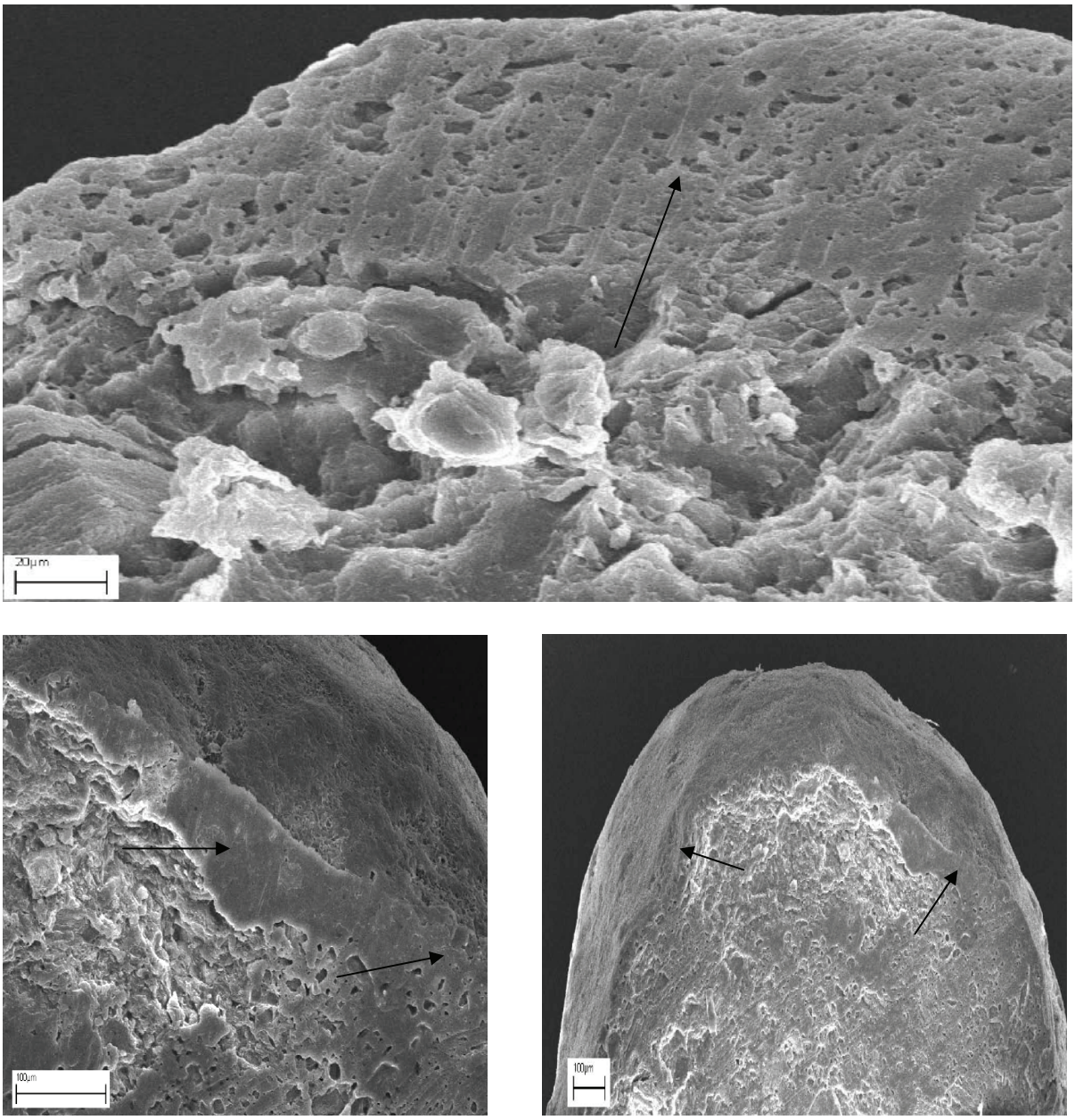


Figure 28: Scanning electron micrographs of a pellet containing 50% L-arginine free base, 50% Dynasan® 114 (batch B1), was taken at an adjusted apparatus magnification of 100 and at an adjusted apparatus magnification of 1000.

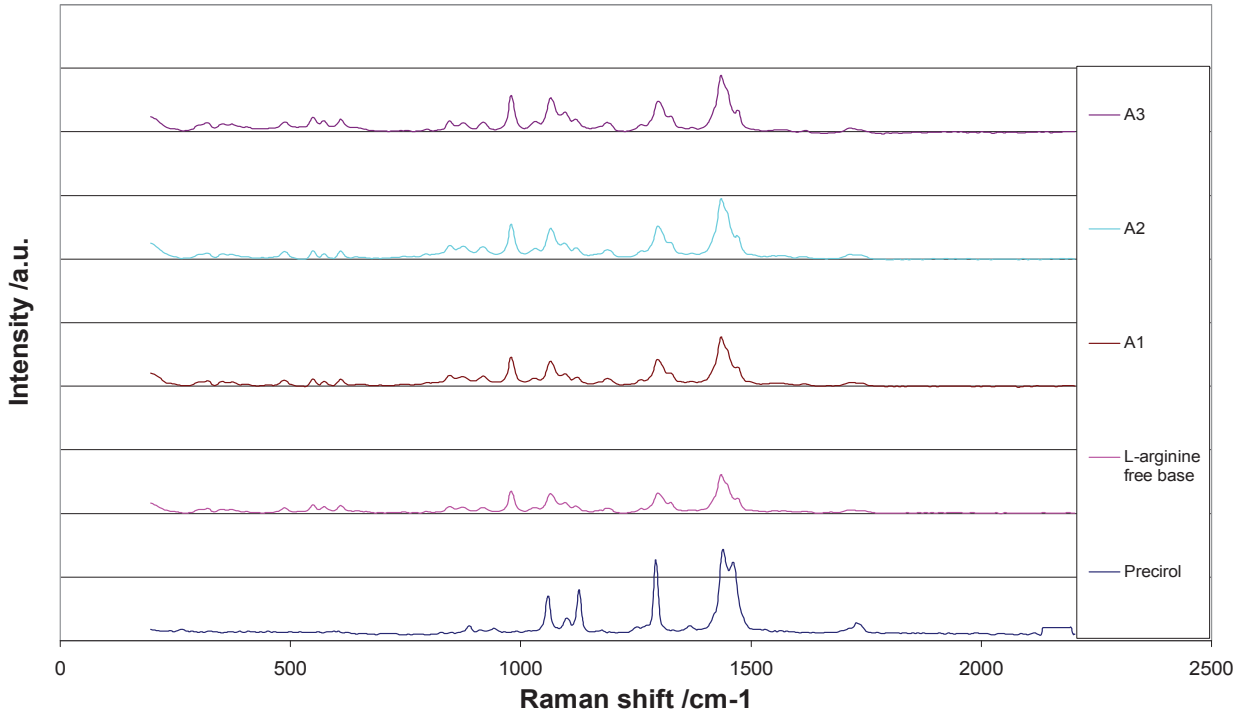


Figure 29: Raman spectra of the A1, A2 and A3 pellet batches, L-arginine and the respective excipients.

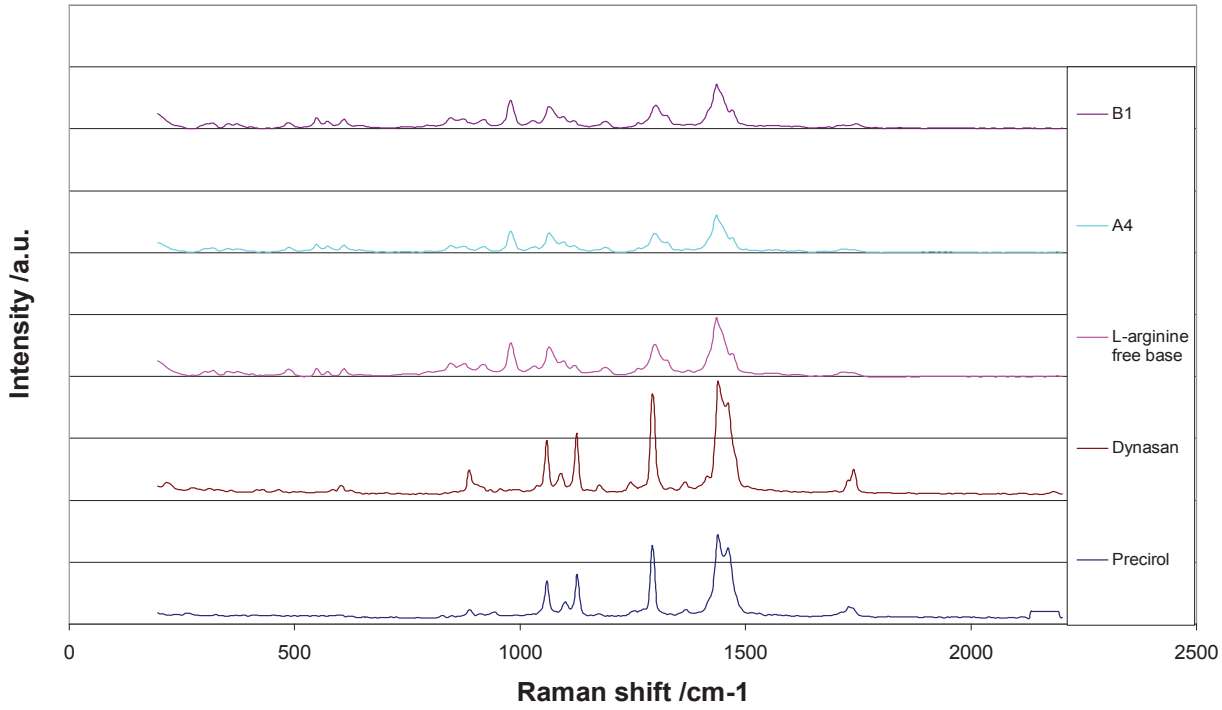


Figure 30: Raman spectra of the A4 and B1 pellet batches, L-arginine and the respective excipients.

The self-coating was found within the scanning electron micrographs of cross-sections of all pellet batches disregarding of their extrusion/spheronization temperature and conditions used.

The pellet batch B1 with 50% Dynasan® 114 displayed in figure 28 reveals not the same visible layer as the previously described scanning electron micrographs of pellet batches (A1-A4) containing 50% Precirol® ATO 5. This may be related to the nature and composition of the lipid binder, as mentioned above under sections 2.3.4 and 2.4.1. This means using Precirol® ATO 5 as a lipid binder to obtain self-coating spherical pellets was better than Dynasan® 114. The Raman spectra for all pellet batches were measured to see if there are any changes in the crystalline structure of the pellet batches and their respective excipients during extrusion/spheronization process. Raman spectra are presented in figures 29, 30, which show no detectable changes in their peak pattern.

2.4.3 Stability of lipid pellets

The effect of storage at elevated temperature on the dissolution profile of all lipid pellet batches was investigated. Stability testing includes long-term accelerated studies where the pellets are stored at 40 °C in an oven for 6 months. The storage temperature is about 15 °C below the melting points of the lipid binder used, Precirol® ATO 5 and Dynasan® 114. Stability data obtained for the drug substance were used to determine the effect of long-term accelerated stability studies on the drug release from the pellets. Drug release and thermal behaviour were determined after storage at 40°C in an oven for 6 months in order to analyze their stability during the storage influenced by time and stored temperature.

HPLC was used to determine the drug release profile. Frequently, the same HPLC method used to determine the drug release profile, although different sample preparation methods would normally be required. The assay testing was performed by using a single HPLC method. However, full validation of the analytical method is required, the establishment of specificity, linearity and limit of quantification is important

at the earliest stages, since verification of stability hinges on a suitable method for separating degradation products from the active ingredient and at least quantifying the degradation products related to the drug substance.

Pellets of batches A1 and B1 showed no changes in drug release after the storage periods and were compared with the release profile obtained one day after extrusion/spheronization (figure 31). The drug release properties remained stable and significant changes in drug release kinetics could not be observed (p-value > 0.05). DSC measurements demonstrated that there was no relevant change in melting peaks of lipids after 6 months of storage at 40°C, as seen in figures 33, 34.

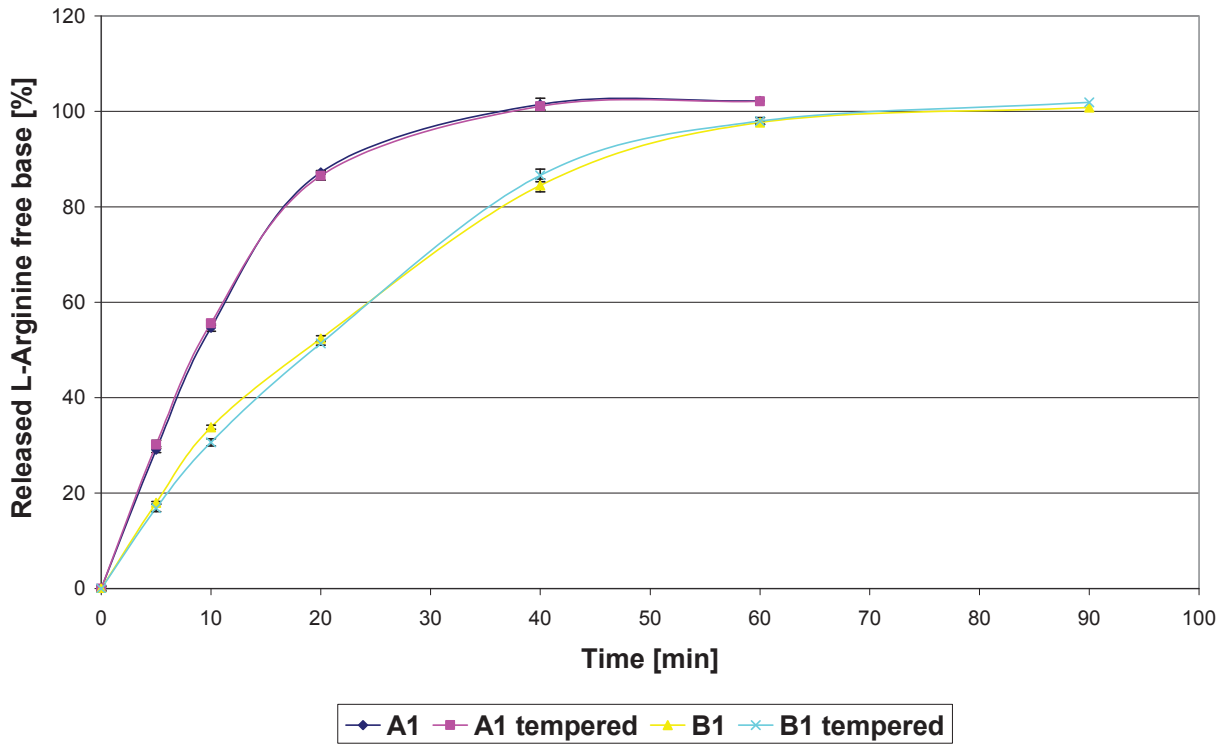


Figure 31: Drug release characteristics after storage under accelerated conditions at 40°C determined by HPLC, dissolution media: Phosphate buffer (mean ± SD, n=6), temperature: 37 °C ± 0.5°C, 75 rpm, basket-Method.

In contrast, pellets of A2, A3 and A4 batches showed changes in drug release after 6 months of storage at 40°C. Comparison the release profile obtained one day after extrusion/speronization (figure 32) significant decrease of the drug release rate was measured after 6 months of storage (p-value < 0.05).

The change in drug release behaviour is supposed to be an effect of different aging processes within the lipid matrix. As it is well known that lipids can undergo changes during thermal treatment [60] and under storage conditions [64]. Aging of the lipid matrix seemed to be a heterogeneous process. The observed changes in drug release were accompanied by a remarkable increase in melting peaks points of the lipid matrix, as seen in figures 35, 36.

Due to their chemical and physical structures, lipids exhibit complex solid-state behaviour including melting, crystallization and physical modifications during processing and even storage. They usually exhibit three different polymorphic forms (α, β´ and β). The relationship is monotropic in most cases: the α-form is the least thermodynamically stable form, β´ is metastable and β is stable, exhibiting the densest packing mode for a lipid. Since the polymorphic behaviour is typically monotropic, each polymorph has its unique melting point. The lipid polymorphic behaviour is quite difficult to predict. Thus, for example, a dosage form produced with a metastable lipid modification and the desired properties may subsequently transform to a more stable one. The result is usually a deterioration of the product's quality and its desired properties including drug release profiles. Moreover, the physical ‘‘ageing” effects during storage must be well understood to avoid any further drug release alteration during storage [145].

The pellets of A2, A3 and A4 batches showed a small amount of polymorphic behaviour after 6 months of storage at 40°C and this is due to the temperature, where some portions of the lipid molten at the surface of pellets during the storage period and directly solidified after the end of storage time when they come in contact at the room temperature. Figure 32, depicts how polymorphism of a lipid during storage stability can influence the dissolution rate of the dosage form after storage.

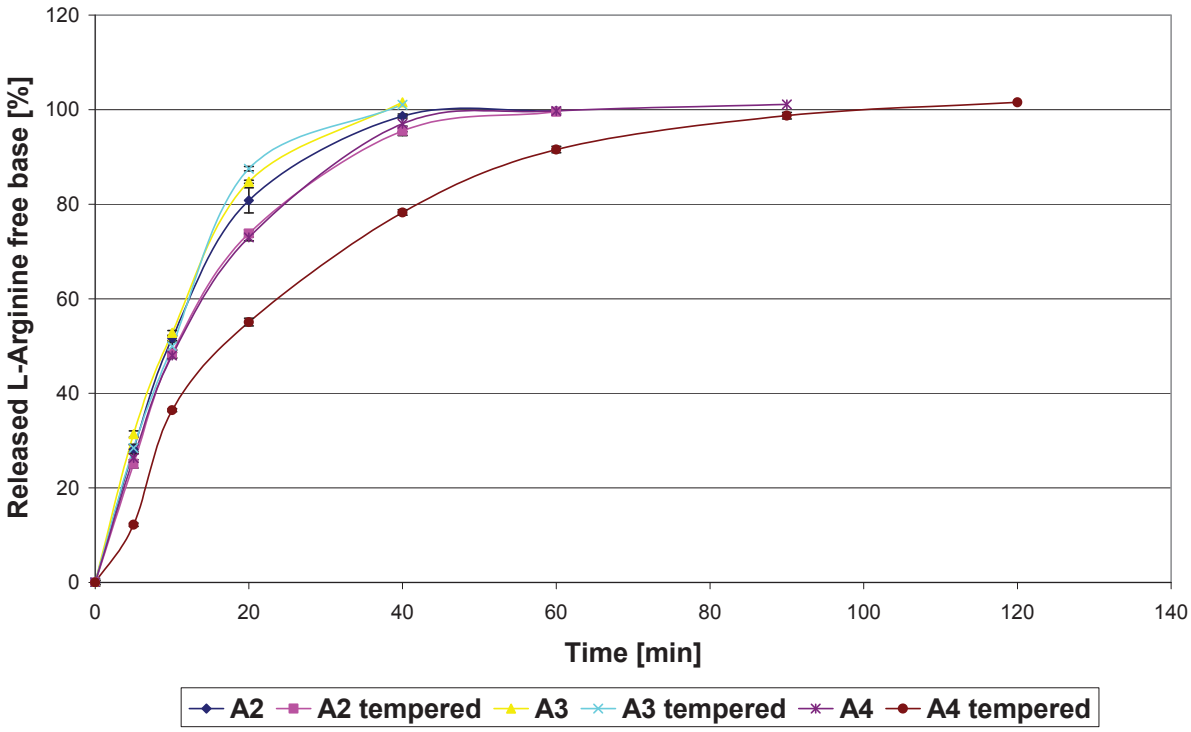


Figure 32: Drug release characteristics after storage under accelerated conditions at 40°C determined by HPLC, dissolution media: Phosphate buffer (mean ± SD, n=6), temperature: 37 °C ± 0.5°C, 75 rpm, basket-Method.

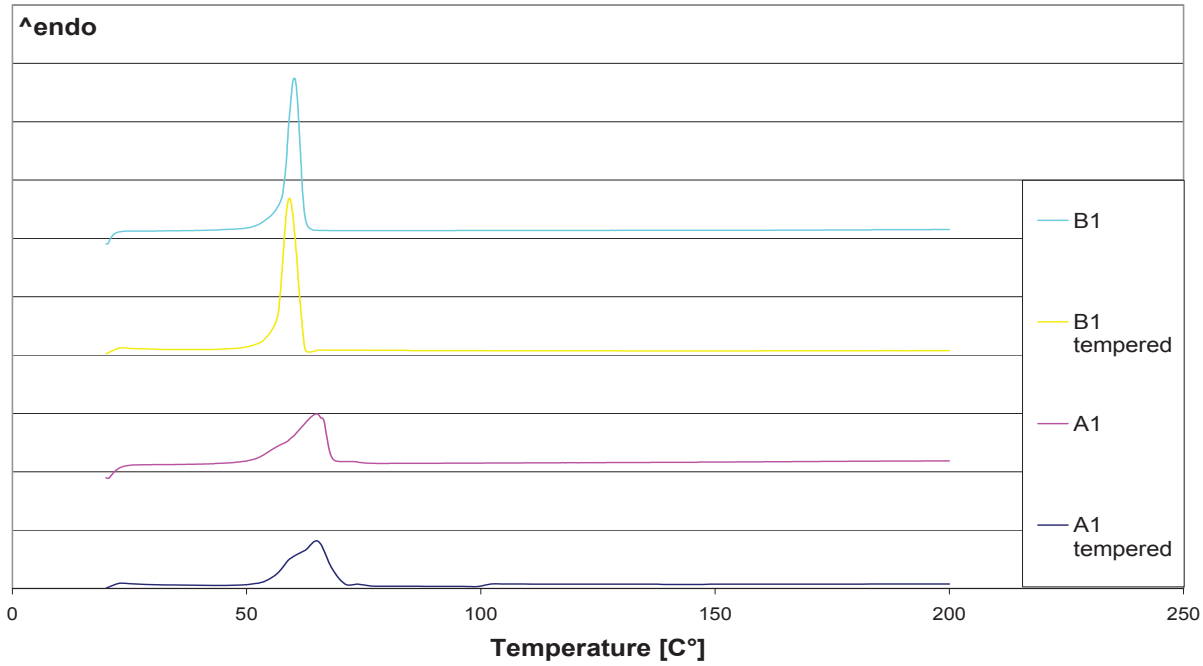


Figure 33: DSC measurements of A1 and B1 batches lipid pellets after storage at 40°C, recorded at a heating rate of 10 K/min, first heating. All signals are scaled to sample weight.

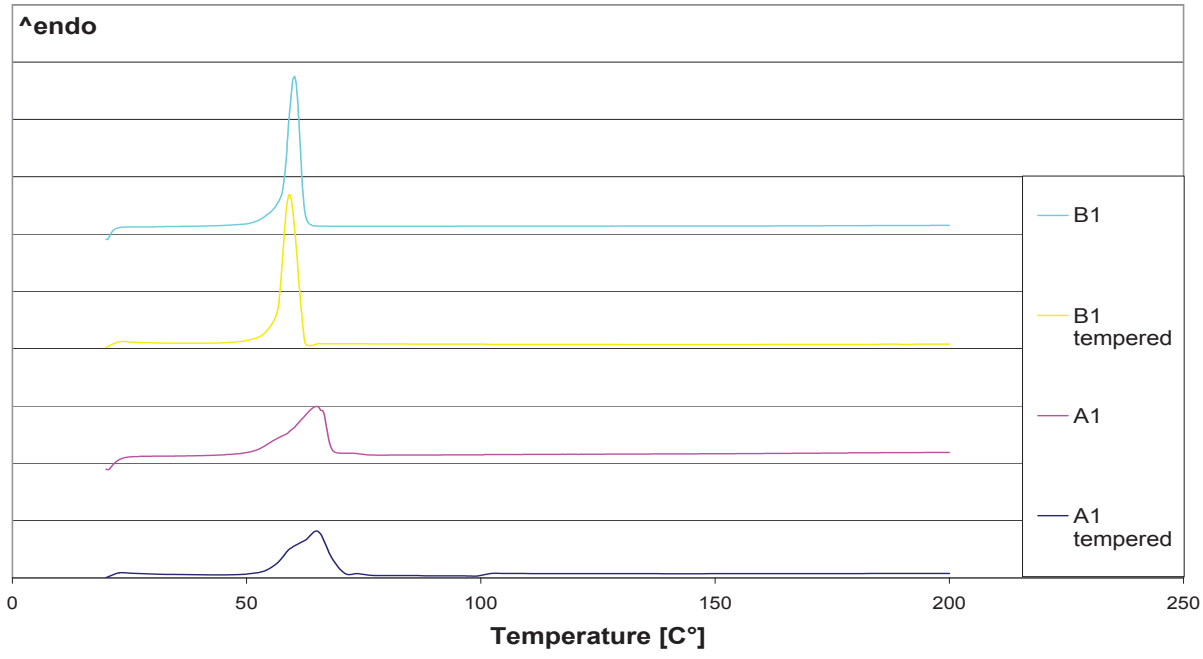


Figure 34: DSC measurements of A1 and B1 batches lipid pellets after storage at 40°C, recorded at a heating rate of 10 K/min, second heating. All signals are scaled to sample weight.

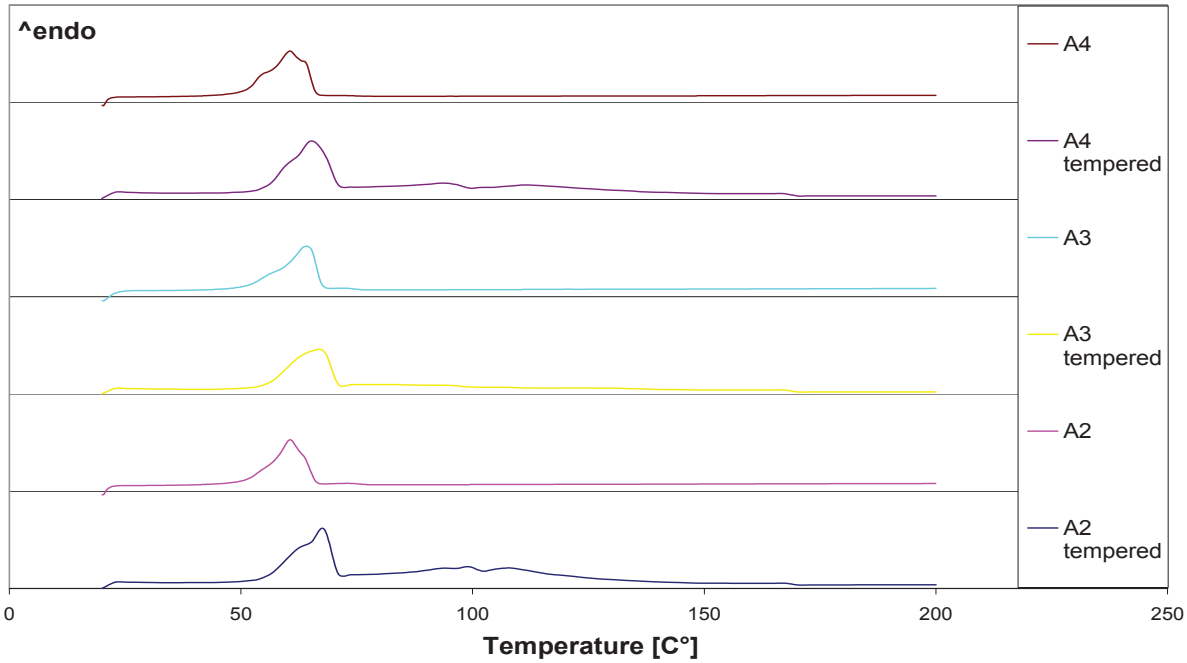


Figure 35: DSC measurements of A2, A3 and A4 batches lipid pellets after storage at 40°C, recorded at a heating rate of 10 K/min, first heating. All signals are scaled to sample weight.

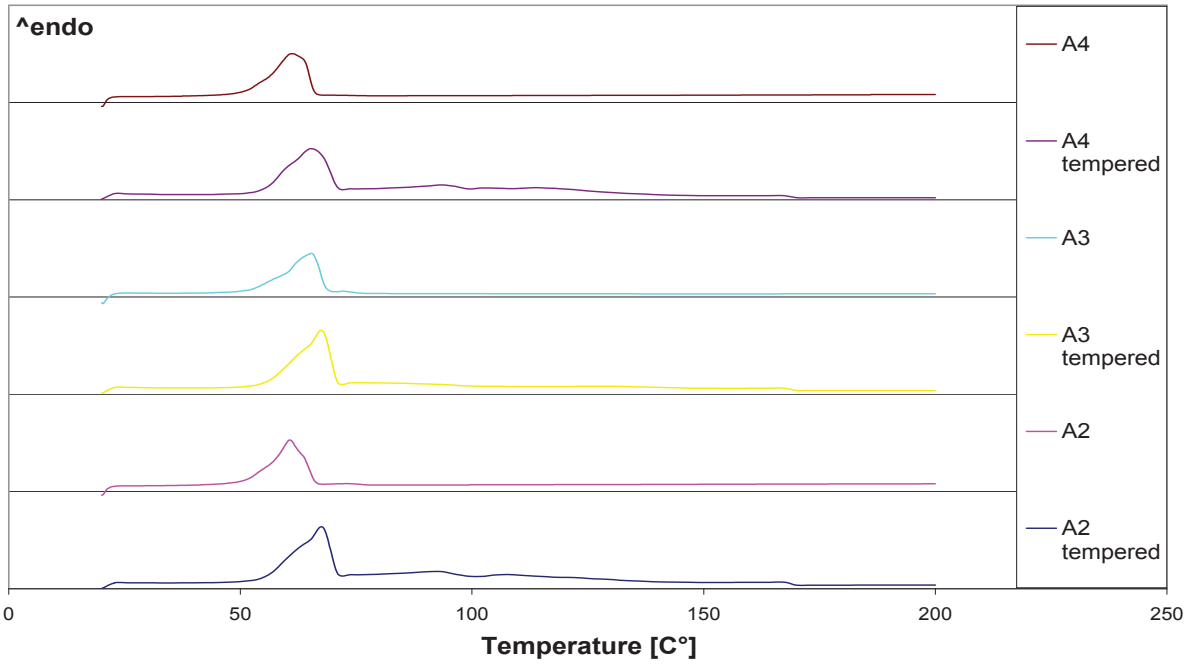


Figure 36: DSC measurements of A2, A3 and A4 batches lipid pellets after storage at 40°C, recorded at a heating rate of 10 K/min, second heating. All signals are scaled to sample weight.

An implication for the dissolution profile can be derived from the surface appearance. To further investigate the discovered effects, scanning electron microscope analysis of lipid pellets before and after dissolution (one day after extrusion/speronization and after 6 months storage at 40°C) revealed interesting differences in the surface appearances. Pellets before dissolution display several different surface features independently from the lipid binder used. In smaller magnifications (40x, 100x; as adjusted at the microscope) no differences are apparent. The lipid pellets have mostly a smooth surface and show a similar surface structure, indicating molten lipids during processing.

At larger magnifications (400x) differences are observed, A1 and A2 batches had lower number of pore-like structures (figures 37, 38), while A3, A4, B1 batches had higher number of pore-like structures and a little flaked surface (figures 39, 40, 41). At magnification (1000x) all pellet batches displayed more like a flaked surface with more pores and needle-like structures, which are probably recrystallised of L-arginine. These features respective of their dependent on the extrusion/spheronization temperature and conditions used.

Pellets of A1 and B1 batches released one day after extrusion/speronization and after 6 months storage at 40°C show a similar surface structure (figures 37, 41). In smaller magnifications (40x, 100x, as adjusted at the microscope) no differences are apparent. Larger magnifications (400x, 1000x) show differences, like a flaked surface with more pores and needle-like structures, which are probably recrystallised L-arginine free base. In clear contrast to this are the surfaces of pellets of A2, A3 and A4 batches after one day after extrusion/speronization and after 6 months storage at 40°C (figures 38, 39, 40). Here even at small magnifications the pellets released after one day of extrusion/speronization look shrunk, deformed and have more cracks than the pellets released after 6 months storage at 40°C. At a closer look none of the previous by described features are present. The surfaces of pellets, which released after 6 months of storage at 40°C, have more a molten-like structure with no visible pores than pellets, which released after one day of extrusion/speronization. It is possible that the lipids or part of it start melting during storage at 40°C after 6 months forming such surfaces. Again, independently from the type of the lipid binder used, the pellet surfaces have the same characteristics and features.

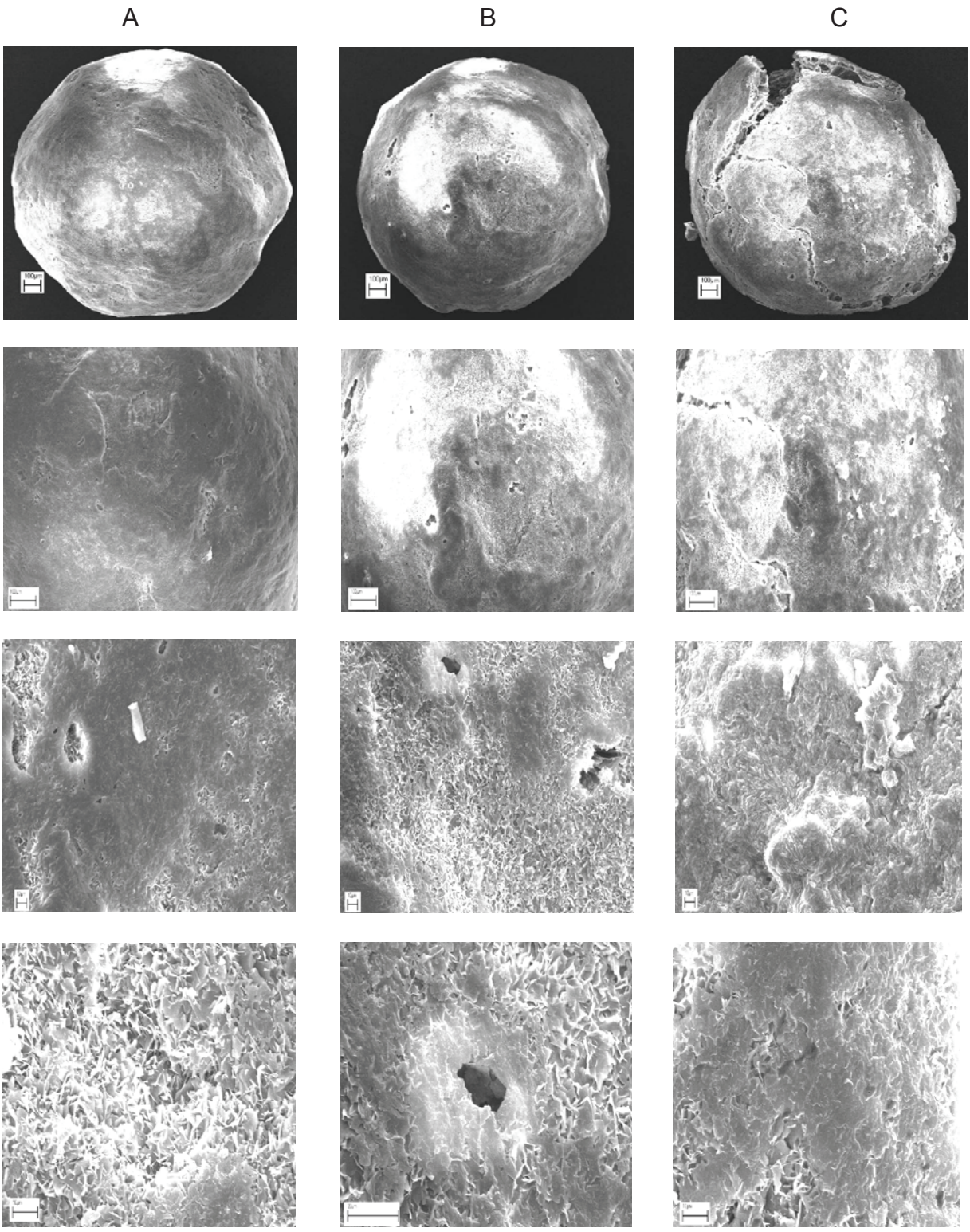


Figure 37: Scanning electron micrographs of pellet batch A1. A: pellet after extrusion/spheronisation, B: pellet one day after extrusion/speronization after dissolution at 37°C, C: Pellet after 6 months storage at 40°C after dissolution at 37°C.

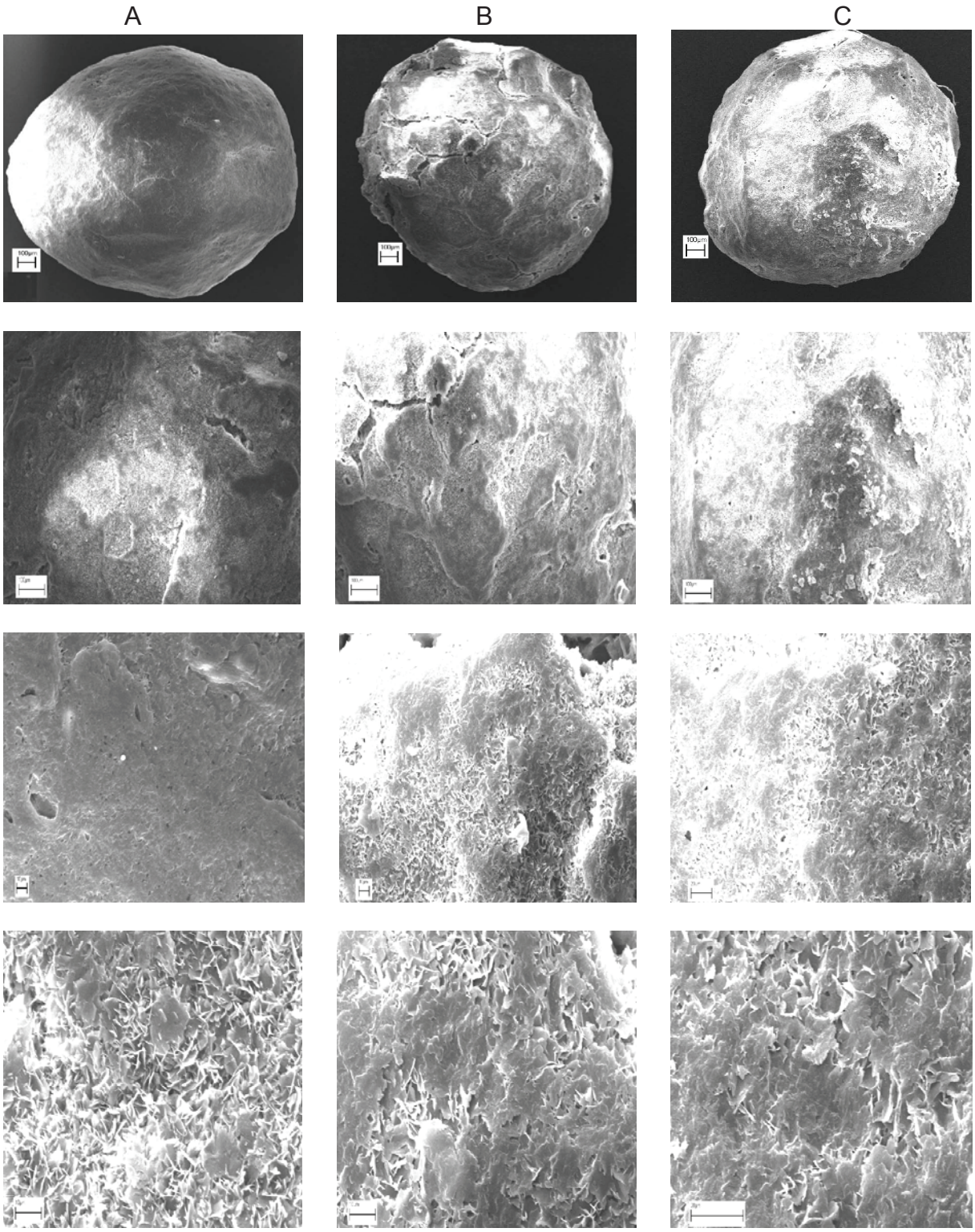


Figure 38: Scanning electron micrographs of pellet batch A2. A: pellet after extrusion/spheronisation, B: pellet one day after extrusion/speronization after dissolution at 37°C, C: Pellet after 6 months storage at 40°C after dissolution at 37°C.

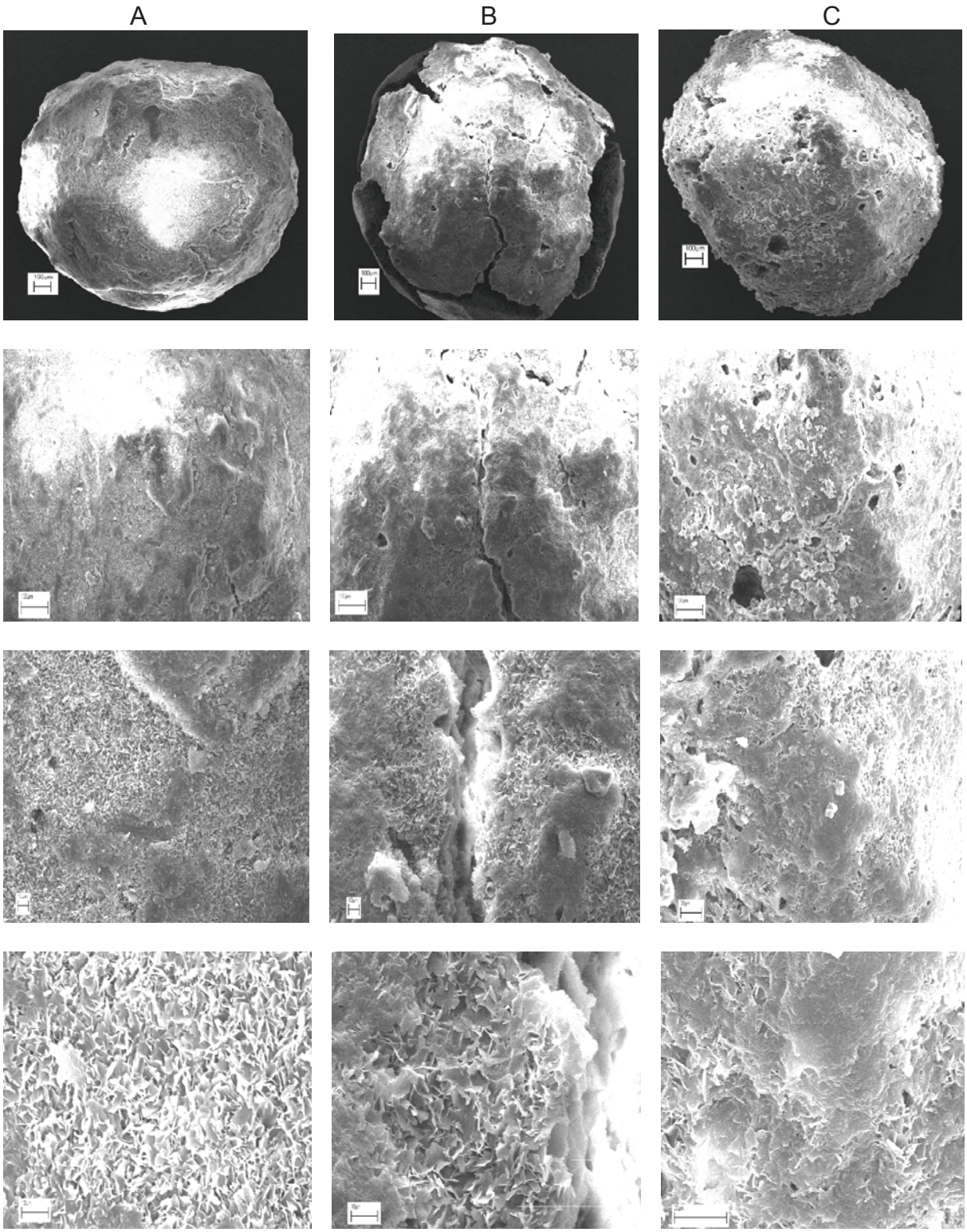


Figure 39: Scanning electron micrographs of pellet batch A3. A: pellet after extrusion/spheronisation, B: pellet one day after extrusion/speronization after dissolution at 37°C, C: Pellet after 6 months storage at 40°C after dissolution at 37°C.

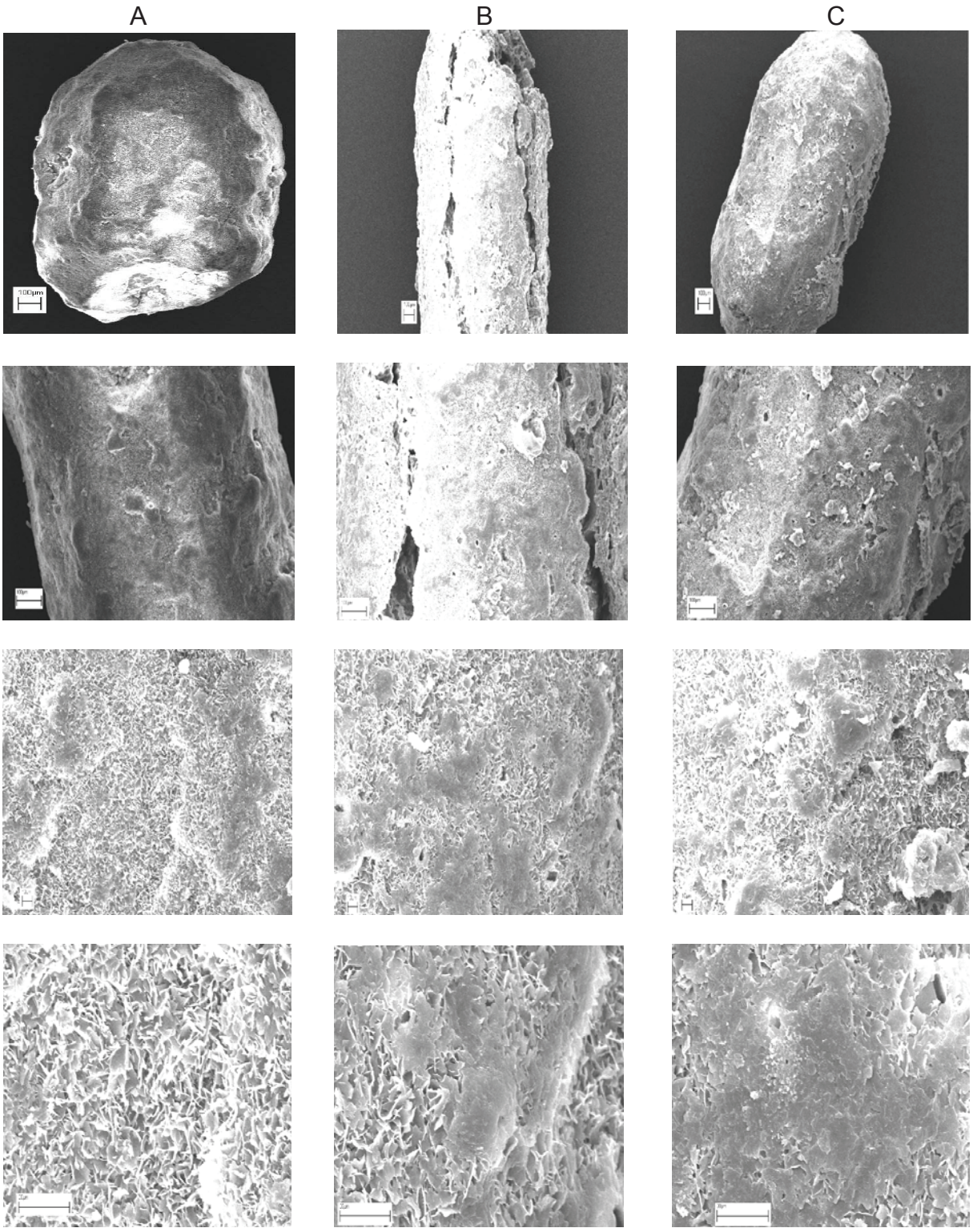


Figure 40: Scanning electron micrographs of pellet batch A4. A: pellet after extrusion/spheronisation, B: pellet one day after extrusion/speronization after dissolution at 37°C, C: Pellet after 6 months storage at 40°C after dissolution at 37°C.

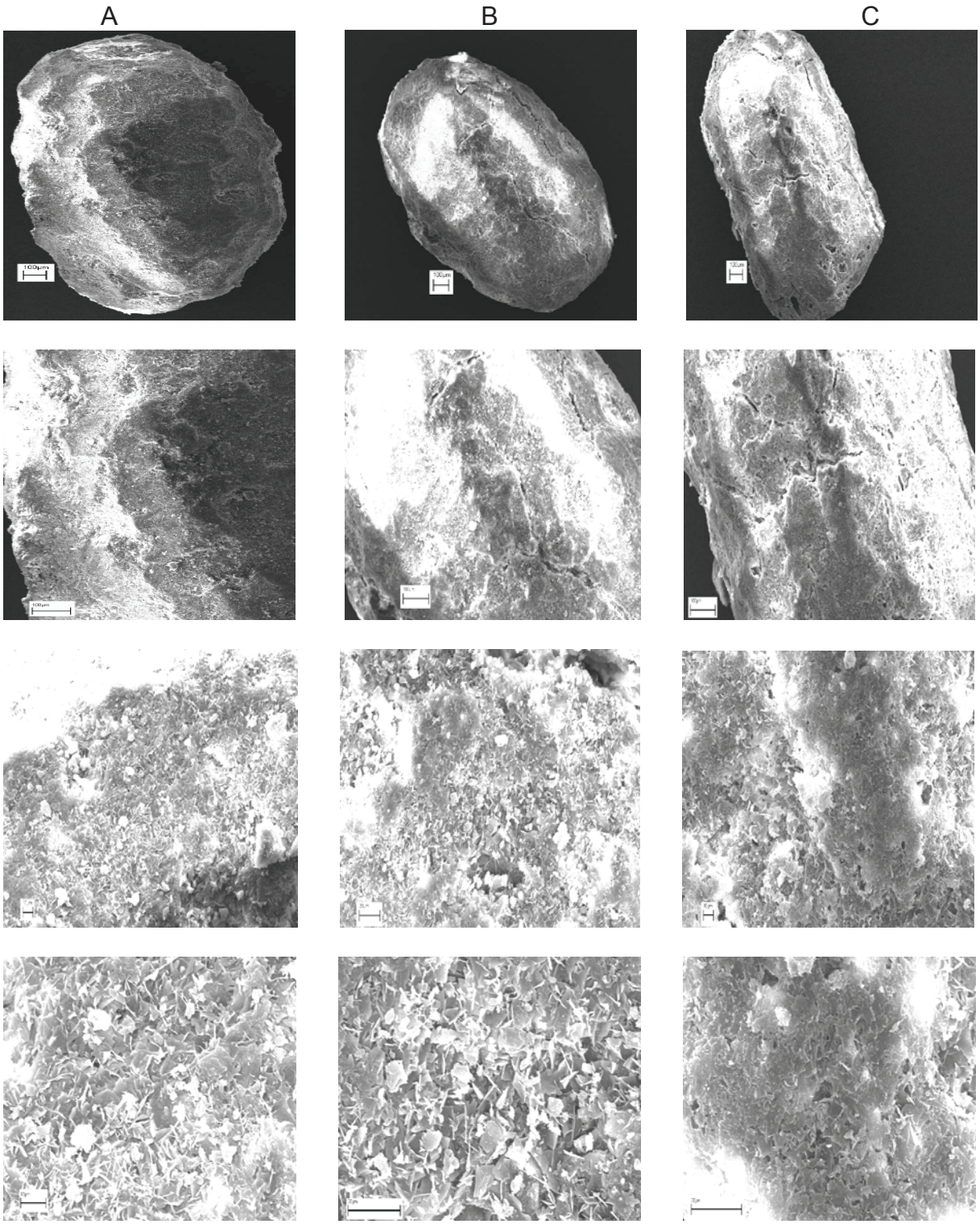


Figure 41: Scanning electron micrographs of pellet batch B1. A: pellet after extrusion/spheronisation, B: pellet one day after extrusion/speronization after dissolution at 37°C, C: Pellet after 6 months storage at 40°C after dissolution at 37°C.

2.4.6 Conclusion

In the present work focusing on L-arginine, solid lipid extrusion did not represent fully satisfying manufacturing technique for sustained release dosage forms. Lipids of different composition could be extruded at temperatures below their melting ranges at solid lipid contents of approximately 50%. Extrusion conditions can have an effect on the lipid matrix structure and on drug release properties. This could be demonstrated for one batch A4, in which drug release rate decreased with decreasing extrusion temperatures.

However, the temperature sensitive nature of the process requires further elucidation of problems with respect to material tempering which results primarily from friction and shear forces which are formulation dependent and difficult to control.
There are differences of surface structures between pellets obtained directly after extrusion/spheronization, pellets one day after extrusion/speronization after dissolution at 37°C and pellets after 6 months of storage at 40°C after dissolution at 37°C. These differences can be seen in the drug release properties during in vitro drug dissolution studies.
Under suitable spheronization process conditions spherical pellets with an aspect ratio of below 1.1 and equivalent diameters of approximately 1.52 mm were obtained from the extrudates. However, the process could be controlled well and the pellets showed almost perfect morphologies. The high spheronization temperatures and the absent of solvents make the described rounding process consisting of extrusion and spheronization an interesting alternative for the manufacture of spherical pellets with low porosity, defined surface area and narrow particle size distribution. The manufactured pellets did not show sustained drug release properties.

By electron scanning microscopy self coating mechanism could be demonstrated which give another example of the most previously discovered principle. The mechanism is a new and can be explained by the production process. To achieve spherical pellets, the respective extrudates are rounded in a spheronizer, which is heated to temperatures of approximately 5°C below the melting range of the lipids.

During this process, some parts of the lipid binder melt due to the heat of the spheronizer's wall and frictional forces in the pellet bed. A separation of parts of the molten lipid binder and the solid crystalline L-arginine takes place. After discontinuation of the spheronizing process molten lipids at the surface of pellets recrystallize. This leads to a layer of lipids of approximately 10 µm thickness around the core of the pellets which acts as a diffusion barrier. However, by this self coating layer around each pellet we could not obtain a sustained release profile for L-arginine, because of the highly water solubility of L-arginine.

Therefore, a further coating step is required by using a water insoluble polymer to obtain sustained-release pellets for L-arginine.

3. Taste assessment

3.1 Taste analysis of different liquid formulations

Taste has an important role in the development of oral pharmaceuticals, with respect to patient acceptability and compliance, and is one of the prime factors determining the market penetration and commercial success of oral formulations, especially in paediatric medicine [141]. Hence, pharmaceutical companies invest time, money and resources into developing palatable and pleasant tasting products and companies adopt various taste-masking techniques to develop an appropriate formulation. Taste assessment is one of the important quality-control parameter for evaluating taste-masked formulations. Any new molecular entity, drug or formulation can be assessed using in vitro or in vivo methods for taste. In vivo approaches include human taste panel studies, electrophysiological methods and animal preference studies. Several innovative in vitro drug release studies utilizing taste sensors, specially designed apparatus and drug release by modified pharmacopoeial methods have been reported in the literature for assessing the taste of drugs or drug products. The multichannel taste sensor, also known as the electronic tongue or e-tongue, is claimed to determine taste in a similar manner to biological taste perception in humans. Furthermore, such taste sensors have a global selectivity that has the potential to classify an enormous range of chemicals into several groups on the basis of properties such as taste intensities and qualities [141].

If perception of component tastes in mixtures requires intentional analysis, this analysis may be affected by age. Thus, perceptual development beyond infancy is often characterized as the development of perceptual-attentional skills. In preschool years, children are often biased to process stimuli holistically rather than analytically. However, analytic skills in perceptual tasks continue to become more focused until late childhood.

Electronic tongues are relatively novel analytical tools in pharmaceutical analysis. They consist of a multichannel taste sensor system and an electronic data management system to predict the human taste of liquid formulations.

As organoleptic aspects of pharmaceutical formulations are an important factor in drug development the electronic tongue is a possible method for the rational development of new drug formulations without the need of human taste panels. Electrochemical sensors may be influenced by the pH and ionic strength of the sample.

3.2 Taste analysis of primary formulations

L-arginine is supposed to have umami and bitter taste [96]. The palatability of both L-arginine as a free base and as a monohydrochloride salt was tested by formulating simple solutions, to select one of them for the further formulation development. The taste of the L-arginine as free base was very umami and bitter with unacceptable odour while the taste of the L-arginine HCL was also very umami and bitter, but the bitterness was less than of L-arginine as a free base and odourless. The umami and bitterness intensity of both was increased by increasing the concentration of both. Simple solutions of both L-arginine as a free base and as a monohydrochloride salt were prepared by using different sweetening agents and taste masking agents, to see the effect of those agents on the taste of both. The umami and bitterness of L-arginine HCL was decreased, but of L-arginine free base was the same did not respond to those agents. Therefore, L-arginine HCL was selected to be the active ingredient instead of the L-arginine free base. Furthermore, the salt form of the active ingredient is the best one from the biopharmaceutical point of view.

Trying to use other different types of inactive ingredients like sweetening agents, flavouring agents and other substances to see their effect on the taste of L-arginine HCL.

Therefore, six different formulations were formulated (table 28) and their taste were analysed in vitro by using taste sensing system SA402B, the respective labelling for the tested formulations is displayed as used in the following figures. The formulations were analysed by the electronic tongue.

Table 28: Labelling of the six formulations tested with taste sensing system SA402B.

Component	A %w/v	B %w/v	C %w/v	D %w/v	E %w/v	F %w/v
L-arginine HCL	10	-	10	10	10	10
L-arginine free base	-	10	-	-	-	-
Masking flavour 501521T	-	-	0.2	-	-	-
Sucralose	-	-	0.05	0.05	0.05	0.05
Masking flavour TP1000	-	-	-	0.2	0.2	-
Strawberry flavour	-	-	-	0.2	-	-
Lemon flavour	-	-	-	-	0.2	-
Glycerin	-	-	-	-	-	10
Purified water (ml) to	100	100	100	100	100	100

3.2.1 Insent taste sensing system SA402B

The taste sensing system SA402B is equipped with 8 sensors with different characteristics evaluate the taste by using 2 kinds of sensor outputs, relative value and CPA value. Each sensor has been developed in order to responds selectively to one taste quality. So various taste sensations can be evaluated by this system. In measurement of taste of those six formulations, 8 sensors were used and 11 kinds of taste information were applied. Table 29, shows the properties of those sensors.

As each taste sensor has been developed to selectively respond to similar taste. Such characteristic is called global selectivity. But there are some interfering substances for taste sensors. It is rather difficult for the taste sensor to detect non-charged substance. Table 30, shows a list of detectable interfering substances on taste information. Basically, artificial sweeteners such as aspartame and asesulfame-K have strong sweet taste and aftertaste from sweetness. So the sensor C00, AE1, AC0 and AN0, which are sensors for bitterness and astringency, can give prolonged taste information, which might be appropriate for aftertaste prediction. As a result, the artificial sweetener can be the interfering substance on the sensors.

Table 29: Property of sensors of taste sensing system SA402B (Intelligent Sensor Technology, Atsugi, Japan).

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Sensor	Relative value	CPA
C00	Bitterness	Aftertaste from bitterness
AE1	Astringency	Aftertaste from astringency
GL0	Sweetness	(Not detected)
AAE	Umami	Substance from umami
CA0	Sourness	(Not detected)
CT0	Saltiness	(Not detected)
AC0	(Not detected)	Basic bitterness 1
AN0	(Not detected)	Basic bitterness 2

Table 30: List of detectable and interfering substances.

Taste information	Detectable substance	Interfering substance
Bitterness Aftertaste form bitterness	Iso-a-acid	Acesulfame-K, Saccharin-Na, SDS
Astringency Aftertaste from astringency	Tannic acid, Catechin, Chlorogenic acid,	Acesulfame-K, Saccharin-Na, SDS
Sweetness	Sugar, Sugar alcohol, Acesulfame-K	Sour substance
Umami Substance from umami	All sour taste (inorganic acid, organic acid)	High concn. of astringent substance
Sourness	MSG, IMP, GMP	None
Saltiness	NaCl, KCl	Acesulfame-K, Astringent substance
Basic bitterness1	All hydrochlorides (quinine, etc)	Saccharine, Ca^{2+}
Basic Bitterness2	All hydrochlorides (quinine, etc)	Aspartame, Ca^{2+}

The sensor GL0 for sweetness, which is under development, needs a different procedure for the measurement from the normal procedure. So using of this sensor needs to measure after each sensor is attached to the sensor support head. Therefore, 3 replications of measurements were required because in this measurement 8 kinds of sensors were used.

Figure 42 shows radar chart with 11 kinds of taste information on samples A to F. From this figure can see that the shape of radar chart of sample B is quite different from any other samples. This means that sample B has a predicted specific taste by comparison with any other samples.

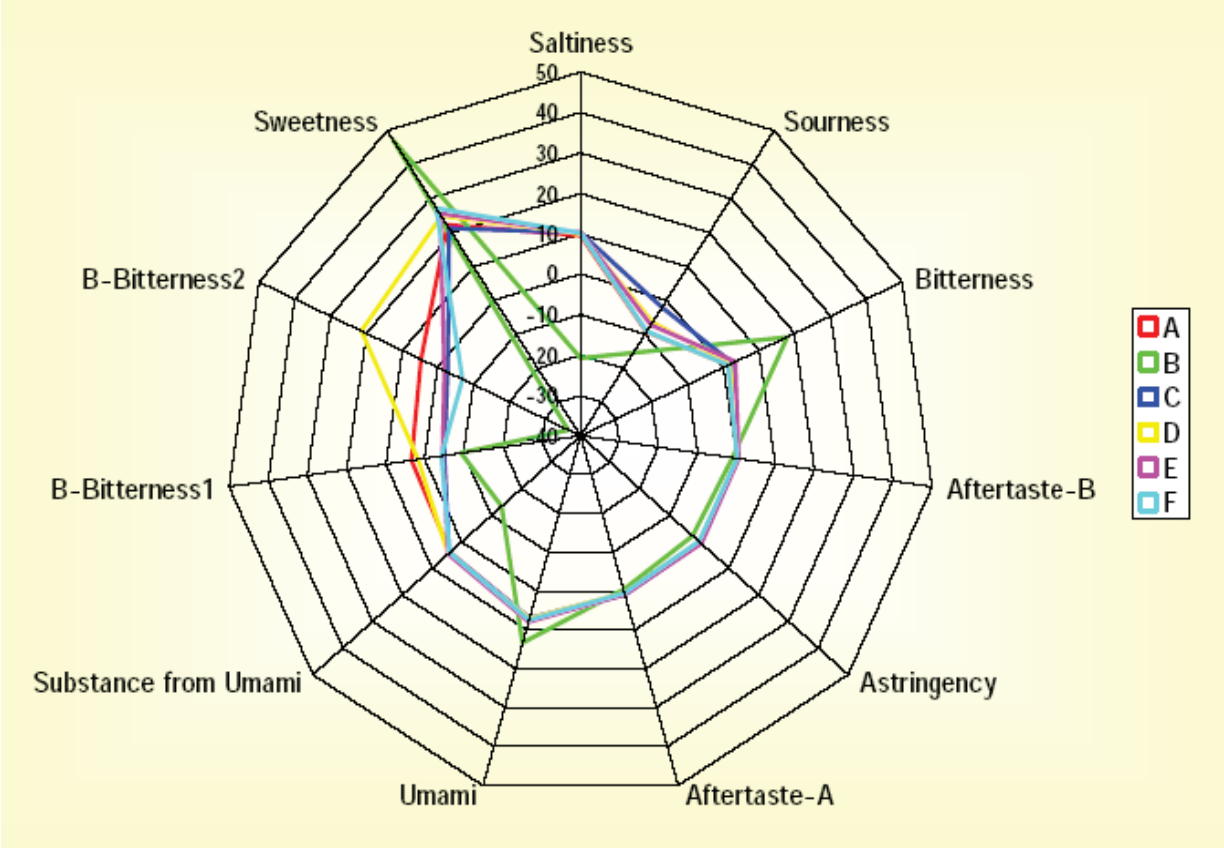

Figure 42: Radar chart with 11 kinds of taste information on samples A to F.

To make the result of radar chart more visible, 2D scatter plot was applied to the result. From figures 43 and 44, it can be clearly seen that sample B is an outlier in these figures. Conductivity and pH of sample B are around 0.3 mS/cm and 11, respectively. The higher pH value of sample B has destroyed the lipid membrane of the sensors, while conductivity and pH of the any other samples are more than 10 mS/cm and around 5 on an average, respectively, figure 48. Therefore, the property of sample B is different from any other sample. So, there is a possibility that taste sensors might not be able to predict the taste of sample B correctly.

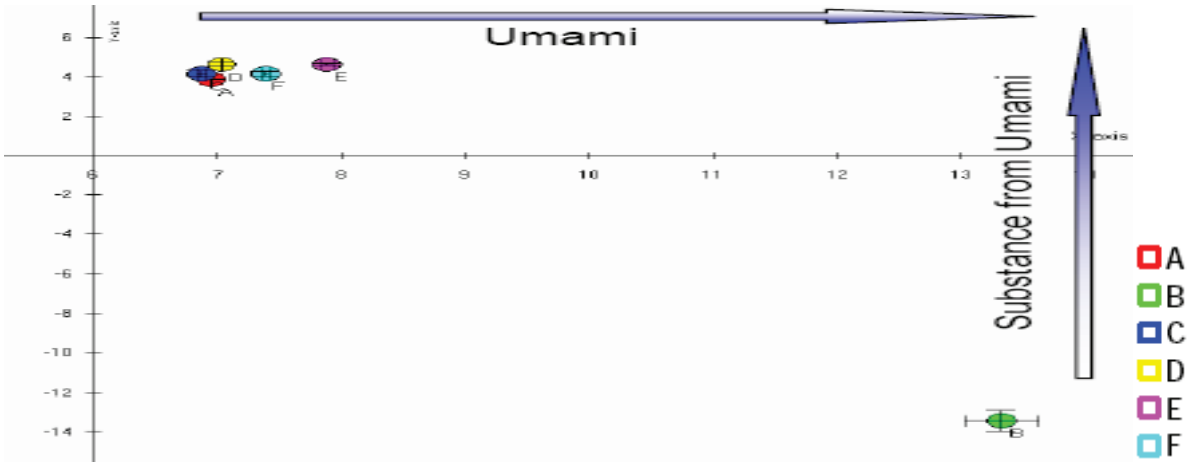

Figure 43: 2D scatter plot (Umami vs. Substance (aftertaste) from Umami).

As shown in figures 43 and 44, it was very difficult to evaluate all the samples because the outlier and any other samples were plotted in the same figure. So to make it easier to evaluate, new measurement of the samples and 2D scatter plot were made without sample B (figures 45, 46, and 47). As a result of taste information without sample B, it is predicted that sample A was the most umami and bitter in these samples and had no any other tastes. Sample C was the least umami and bitter in these samples and had a slight aftertaste from bitterness. Sample D and E were relatively sweet but it had a slight bitter taste and aftertaste from sweetness. Sample F had a slight bitter and aftertaste from bitterness.

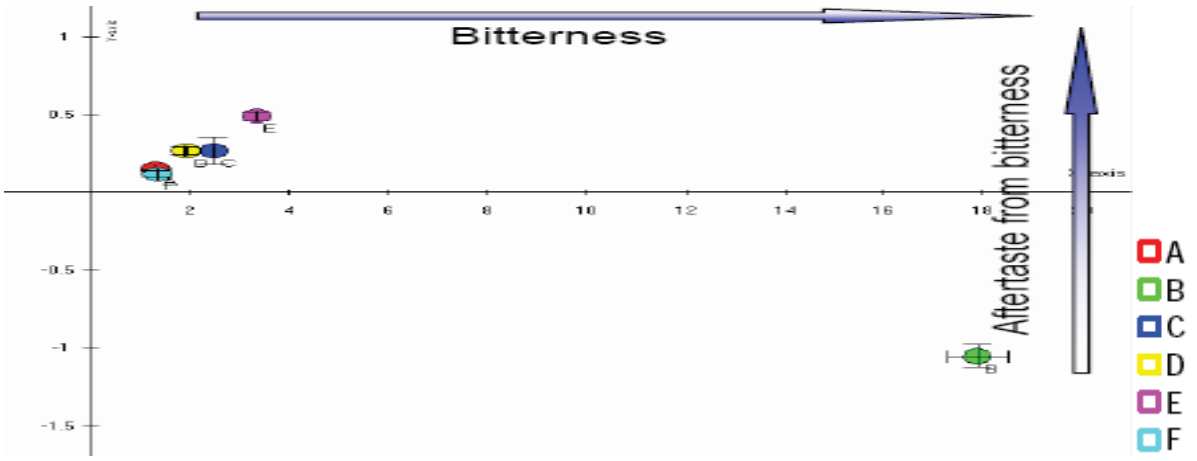

Figure 44: 2D scatter plot (Bitterness vs. Aftertaste from bitterness).

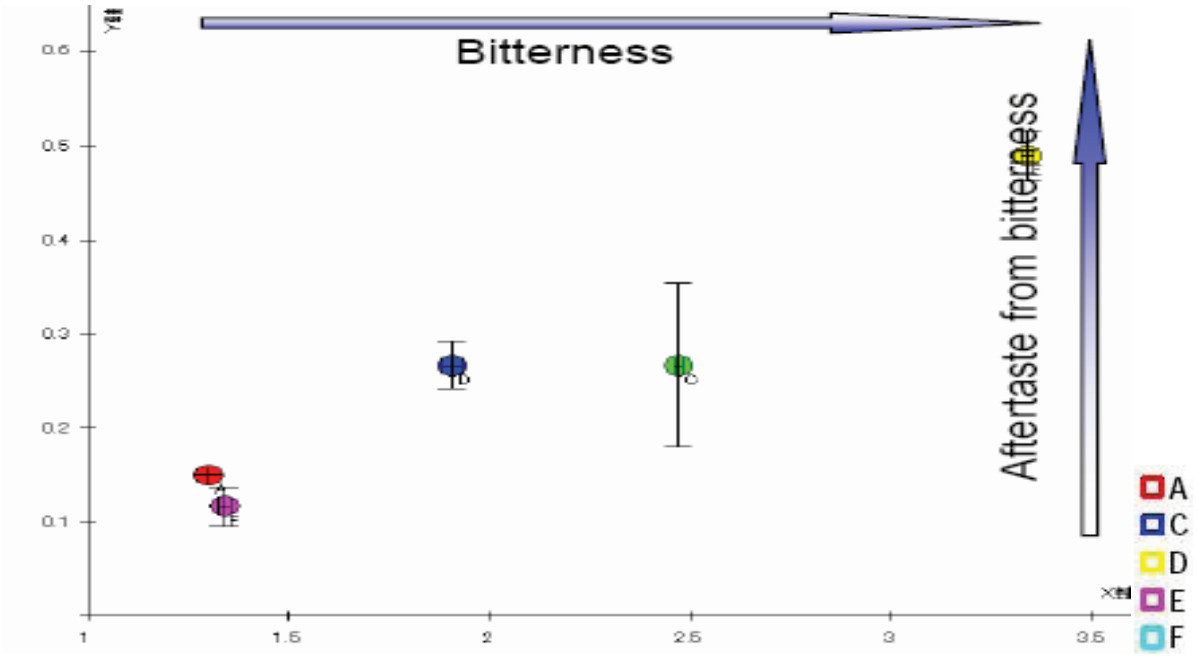

Figure 45: 2D scatter plot (Bitterness vs. Aftertaste from bitterness).

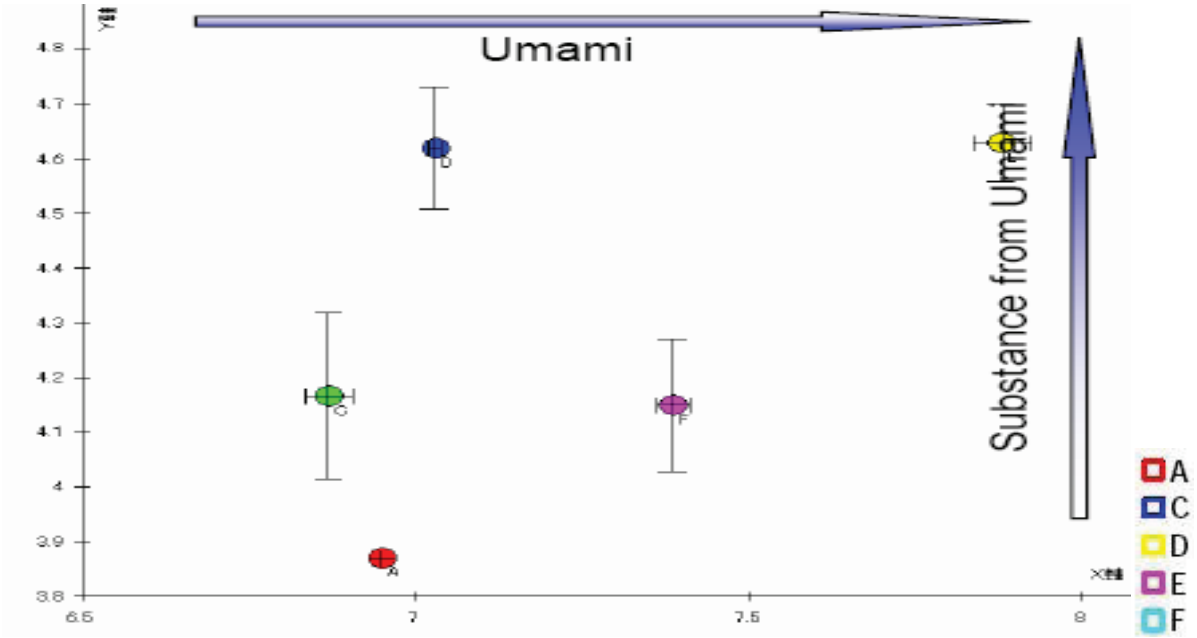

Figure 46: 2D scatter plot (Umami vs. Substance from Umami)

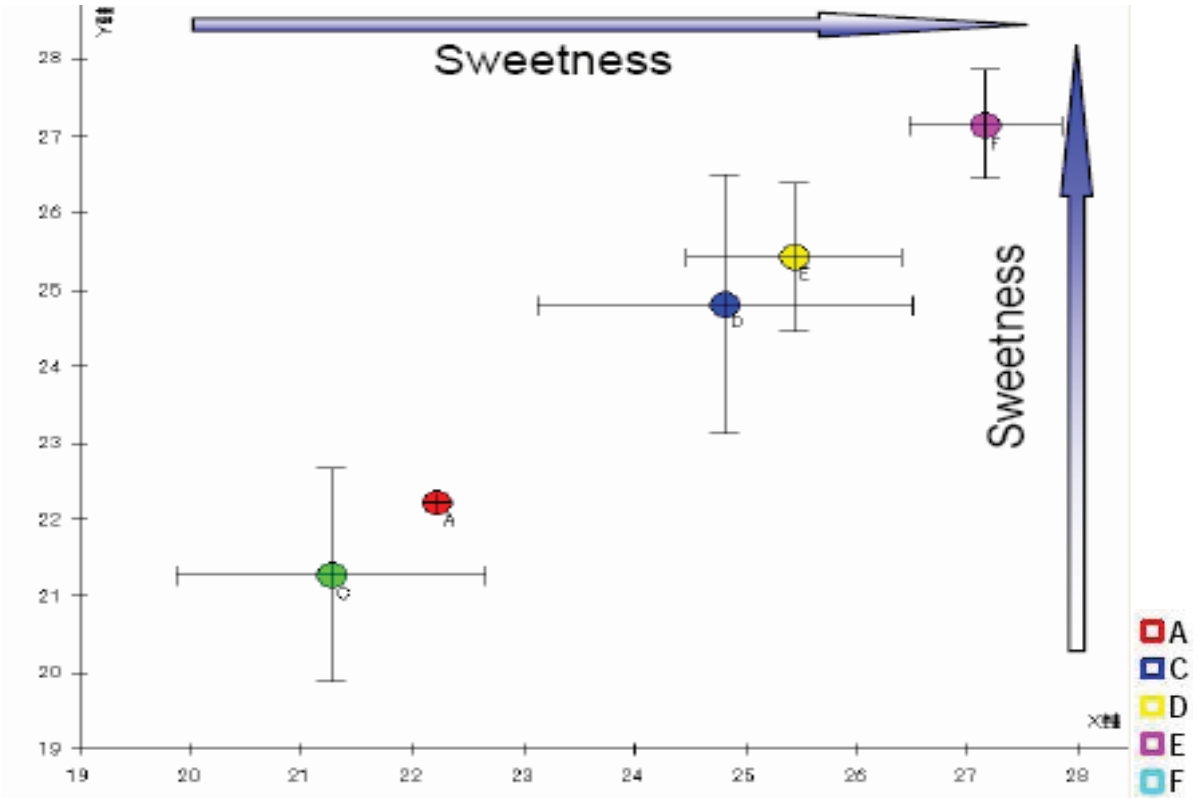

Figure 47: 2D scatter plot (Sweetness)

It was difficult to distinguish the difference between sample C, D, E and F. Also, it was difficult to evaluate the bitterness of samples C, D, and F because these samples had a slight bitterness and a slight aftertaste from sweetness. The primary bitter substance is thought to be L-arginine monohydrochloride, so it should be evaluate the bitterness with taste information (basic bitterness) according to the list in table 29. Also, the sweetness should be evaluated with taste information (sweetness) in figure 47.

According to the list in table 30, the sweetness sensor GL0 detects sugar and sugar alcohols. Of course, sucralose has an extremely strong sweetness. But at least 100 mM concentration is needed for the sensor GL0 to detect this substance. So the concentration of these samples were around 0.6 mM (because the samples were diluted in half), it is thought that sweetness sensor GL0 could not detect the sweetness provided by sucralose.

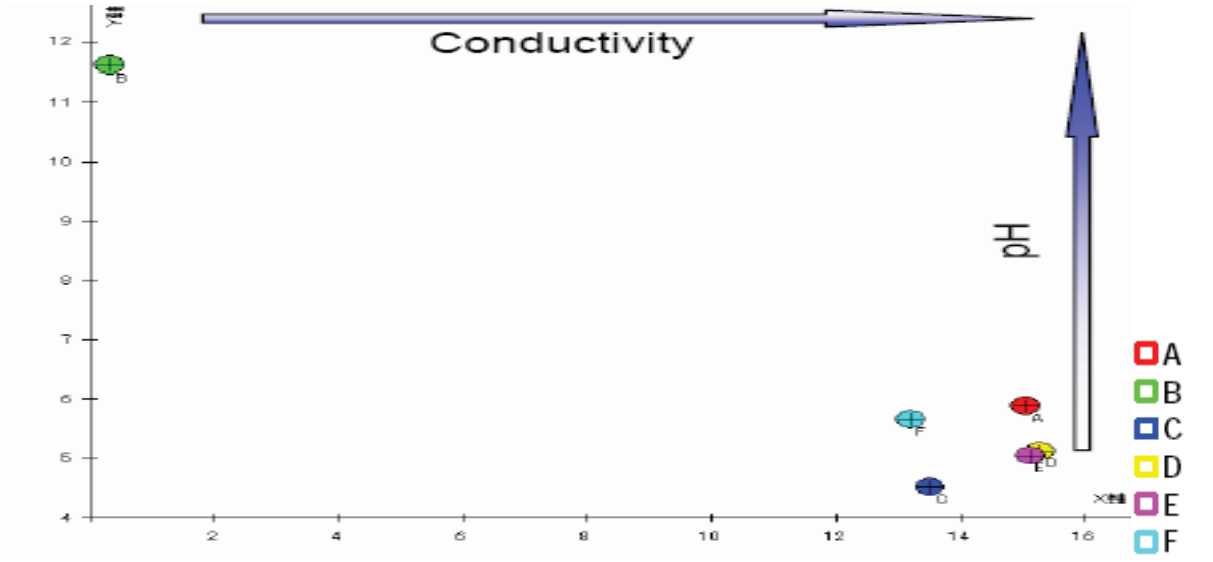

Figure 48: 2D scatter plot (Conductivity vs. pH)

As to the result of samples testing by taste sensor on the samples from A to F, it was found that sample B was an outlier in comparison to any other samples, so it was difficult to predict the taste of the sample B correctly. But, it can get a possibility that taste sensor can evaluate various tastes of the any other samples except the sample B. For this reason, another study was carried out to see the effects of pH and ionic strength on the taste prediction by using taste sensing system SA402B.

3.3 Effects of pH and ionic strength on the taste assessment

The objective of this study was to detect and predict the effects of pH and ionic strength on sensor signals of taste sensing system SA402B of five different types of formulations, table 31, with and without L-arginine HCL as a model drug were evaluated. Before the measurement of the five different L-arginine HCL formulations a calibration was performed using 6 aqueous L-arginine HCL solutions (concentration ranged from 1% to 15%). These calibration solutions were analysed to check the stability and reproducibility of the sensor responses. First, the direct sensor responses [in mV] were analysed, before the data treatment was performed using the insent software. The change from direct sensor responses mV values into taste information values occurs through the insertion of the mV data into an equation to receive the respective taste information values (see Table 40).

Component	F1A,F1, F1wb6.5, F1wb, F1wob.	F2B,F2, F2wb6.5, F2wb, F2wob.	F3C,F3, F3wb6.5, F3wb, F3wob.	F4D,F4, F4wb6.5, F4wb, F4wob.	F5F,F5, F5wb6.5, F5wb, F5wob.	F6E,F6, F6wb6.5, F6wb, F6wob.	F7G,F7, F7wb6.5, F7wb, F7wob.
L-arginine HCL (g)	10 only F1, F1A.	10 only F2, F2A.	10 only F3, F3A.	10 only F4, F4A.	10 only F5, F5A.	10 only F6, F6A.	10 only F7, F7A.
Domiphen bromide (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sodium bisulfite (g)	-	1	-	-	1	1	-
Glycerin (g)	-	-	10	-	-	10	10
Na2EDTA (g)	-	-	-	0.1	0.1	-	0.1
purified water (mL)	to 100	to 100	to 100	to 100	to 100	to 100	to 100
Formulation	F1A	F2B	F3C	F4D	F5E	F6F	F7G
pH	6.50±0.00	6.55±0.05	6.53±0.02	6.50±0.00	6.51±0.02	6.46±0.05	6.50±0.00
Condictivity [μScm^{-1}]	44500.16±0.28	65500.00±0.00	44900.30±0.51	44000.46±0.45	67500.00±0.00	63580.16±0.28	44500.00±0.00
Formulation	F1	F2	F3	F4	F5	F6	F7
pH	5.60±0.08	4.10±0.10	5.40±0.10	5.00±0.05	3.90±0.08	4.25±0.00	4.95±0.05
Condictivity [μScm^{-1}]	44300.16±0.28	45500.00±0.00	44660.23±0.25	43340.03±0.05	50000.00±0.00	47080.33±0.41	43860.00±0.00
Formulation	F1wb6.5	F2wb6.5	F3wb6.5	F4wb6.5	F5wb6.5	F6wb6.5	F7wb6.5
pH	6.50±0.00	6.46±0.05	6.51±0.02	6.50±0.00	6.51±0.02	6.53±0.05	6.46±0.05
Condictivity [μScm^{-1}]	210.00±0.00	16650.00±0.00	225.16±0.28	825.00±0.00	1625.16±0.28	14900.46±0.45	850.30±0.51
Formulation	F1wb	F2wb	F3wb	F4wb	F5wb	F6wb	F7wb
pH	10.76±0.05	7.11±0.02	10.53±0.05	7.43±0.05	7.11±0.02	11.88±0.07	7.35±0.05
Condictivity [μScm^{-1}]	350.00±0.00	22250.00±0.00	590.16±0.28	1050.43±0.40	24450.16±0.28	15000.30±0.51	1050.46±0.45
Formulation	F1wob	F2wob	F3wob	F4wob	F5wob	F6wob	F7wob
pH	5.08±0.07	2.10±0.10	5.53±0.05	4.53±0.05	2.40±0.10	3.05±0.05	4.40±0.00
Condictivity [μScm^{-1}]	210.56±0.49	20000.00±0.00	250.00±0.00	660.63±0.23	17500.20±0.26	16500.16±0.28	640.00±0.00

Table 31: five different types of formulations with their different measured pH and conductivity [μScm^{-1}] ($x \pm s$, $n = 3$).

In this study each sample was assayed in four replications by an electronic tongue (Insent Taste Sensing System SA402B, Japan) equipped with bitterness (C00) astringency (AE1), sourness (CA0), umami (AAE) and saltiness (CT0) detecting sensors.

Exemplary, in figure 49 the sensor responses from all five sensors (umami: SB2AAE, saltiness: SB2CT0, sourness: SB2CA0, bitterness: SB2C00, and astringency: SB2AE1) are displayed for the calibration sample with a concentration of 10% L-arginine HCL (which is equal to the concentration of L-arginine HCL in those measured formulations). Each bar represents one run from the test series. Through this data application it is possible to detect drift in sensor responses, as can be seen with sensor SB2CA0. CPA values are the sensor response to the standard solution immediately after the measurement of the active sample, in between these two measurements the sensors are only cleaned very shortly. This measurement procedure is preformed to detect any delayed sensor signals due to adherence of sample substance to the sensor, indicating an aftertaste of the sample. As a standard procedure, all formulations were measured four times and the first run was omitted in further data handling. To analyse the response from separate sensors, sensor data from one sensor for all measured samples are displayed in a bar chart (figure 50) for the bitterness sensor and all measured samples. The direct sensor response turned more negative with an increase in L-arginine HCL concentration, where as the signal for the aftertaste changed with lower concentrations. This is possible as low concentration of L-arginine HCL is likely to get washed off in the short cleaning steps between relative value measurement and CPA (aftertaste prediction), whereas higher concentrations would need a longer cleaning process and therefore are detectable in the CPA procedure.

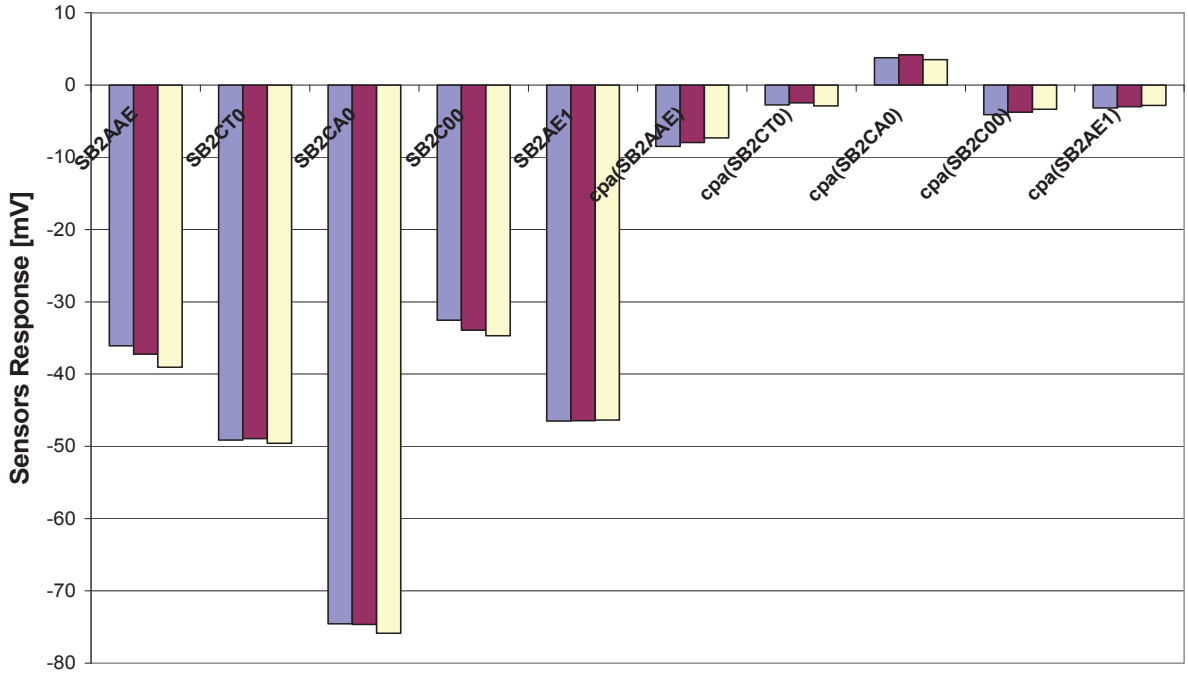

Figure 49: Sensor responses of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for one calibration sample (10% L-arginine HCL). On the right the “aftertaste” sensor signals (CPA) are displayed.

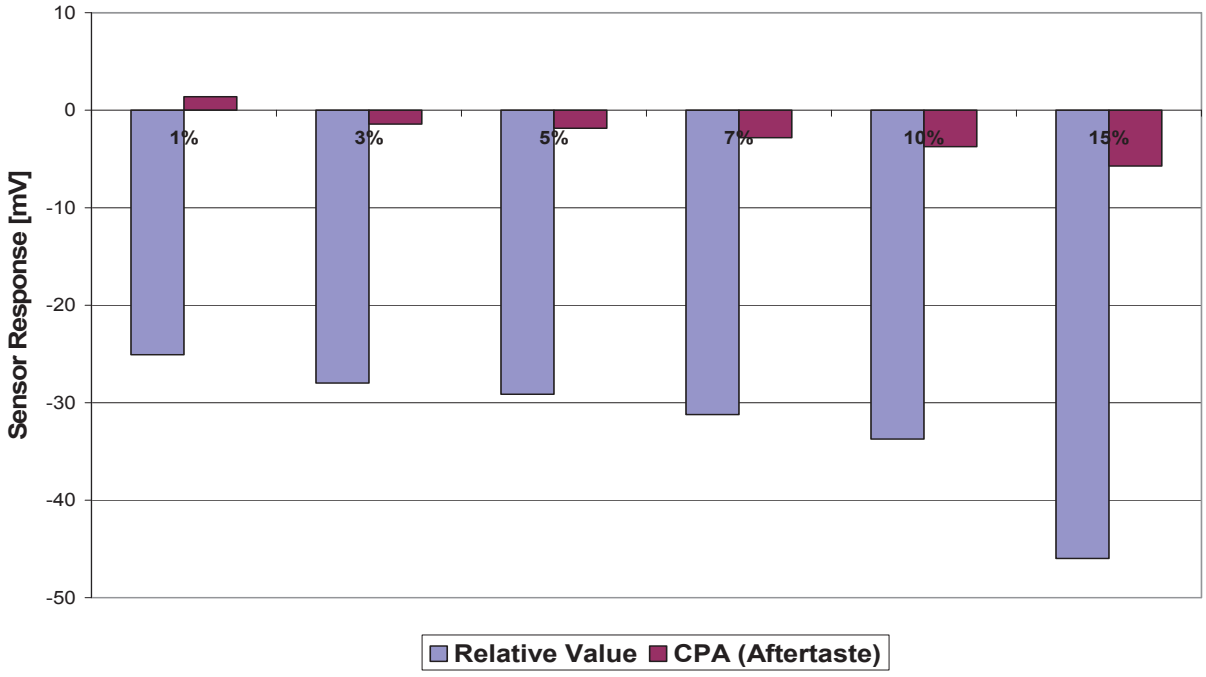

Figure 50: Sensor responses (incl. CPA values) from one sensor (SB2C00) for all calibration samples (1% to 15%), n=3.

As L-arginine is an umami and bitter tasting drug, the raw data of SB2AAE are displayed in Figure 51. As can be seen from the graph, the umami sensor signal is linear in its response to the L-arginine HCL concentrations throughout the calibration range. The bitterness sensor SB2C00 in figure 50, shows a small scattering, especially with low concentration of L-arginine HCL, but is able to detect differences between all samples.

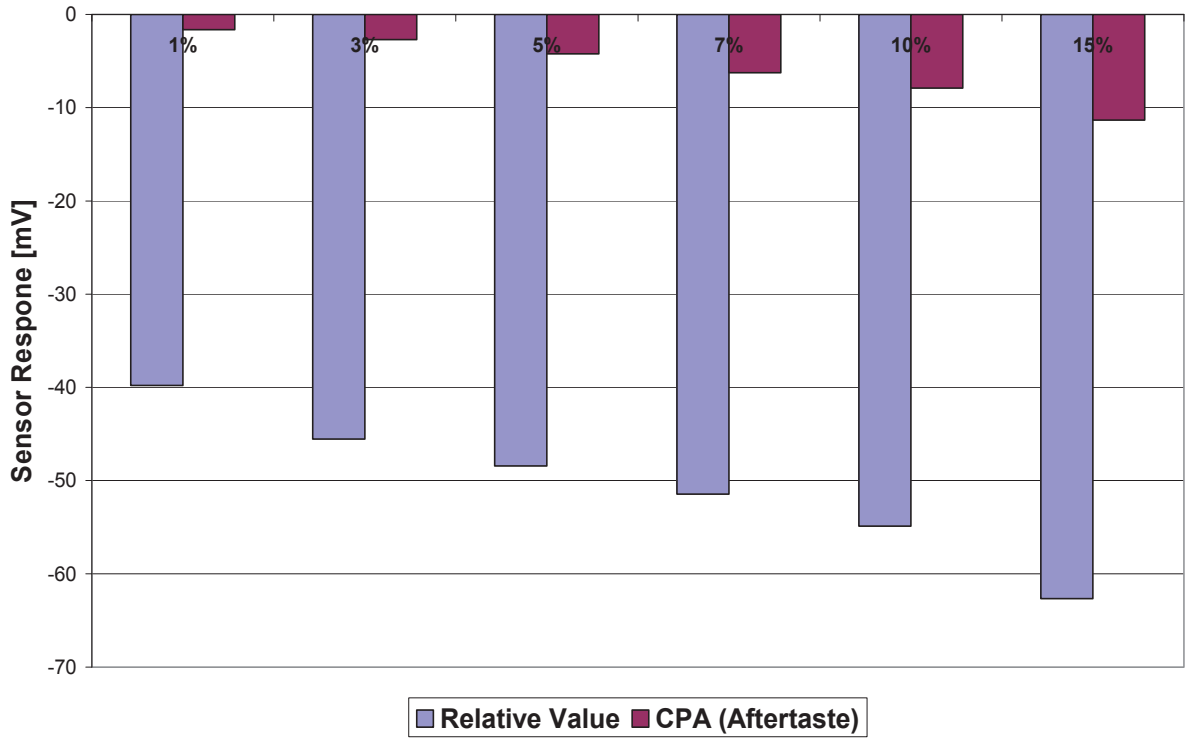

Figure 51: Sensor responses (incl. CPA values) from one sensor (SB2AAE) for all calibration samples (1% to 15%), n=3.

The data obtained from the taste sensing system SA402B in the terms of repeatability and objectivity were assessed by using solutions contain different concentrations of L-arginine monohydrochloride. The sensor signals and not the calculated taste information were used because the correlation was very well even for the direct sensor signals (figures. 52, 53).

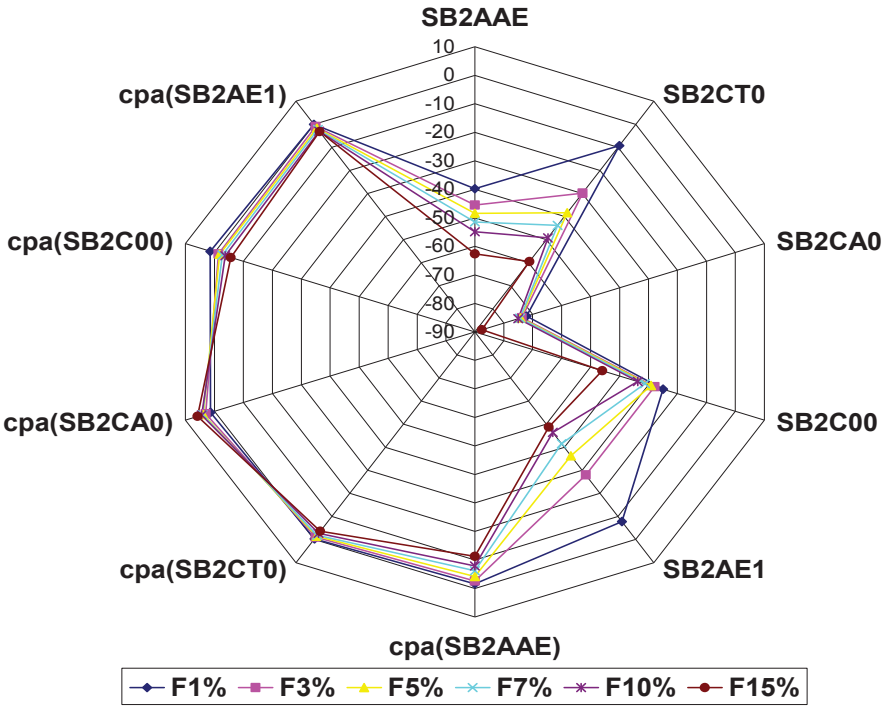

Figure 52: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for all calibration samples of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

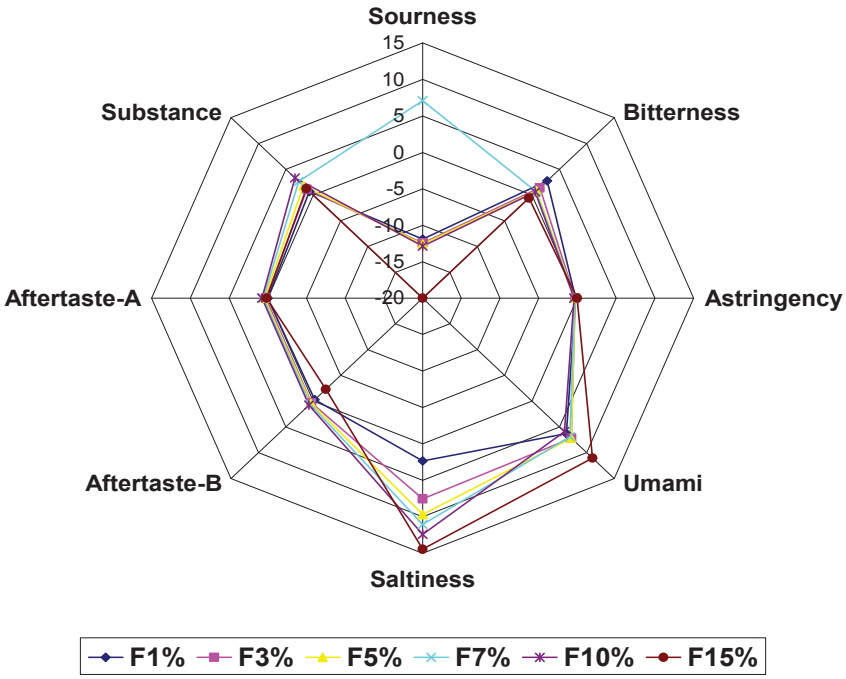

Figure 53: Radar chart of sensor taste information of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for all calibration samples of L-arginine HCL.

After calibration confirmed the ability of e-tongue to measure and detect L-arginine HCL in the estimated concentration range the aqueous formulations were investigated using the same set-up. Even though the Insent electronic tongue is capable of performing measurement of samples without the respective placebo formulations.

The formulations were compared by using F10% formulation as a reference formulation for each measurement which is 10% solution of L-arginine HCL with pH 5.55±0.05 and conductivity of 43250±0.0 [μScm^{-1}] (x ± s, n =3), to see the effect of different pH and ionic strength on sensor signals of those formulations, as can be seen in figures 54, 55, 56, 57, 58, 59, 60.

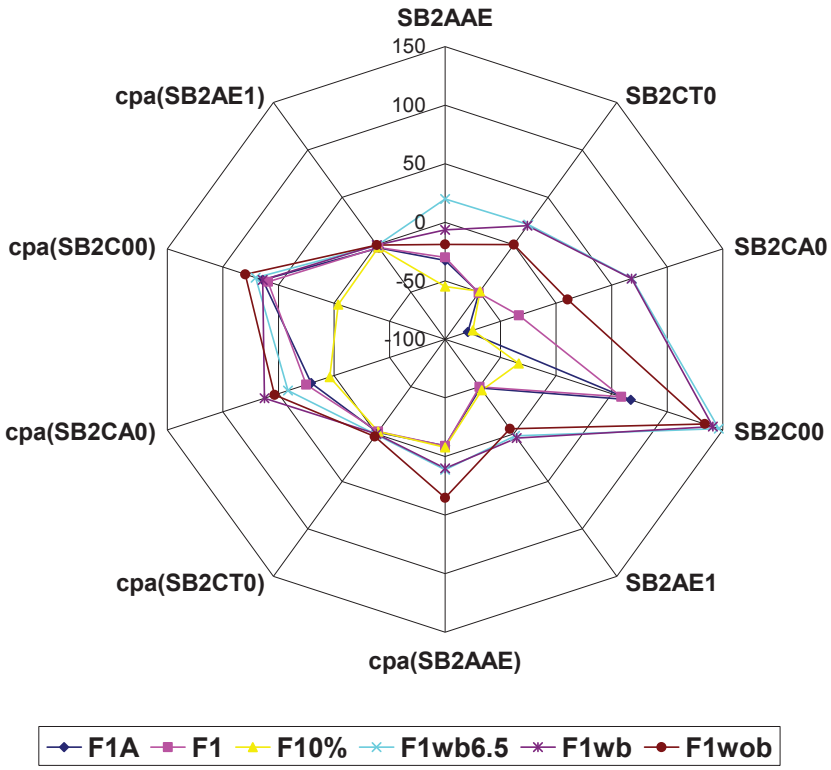

Figure 54: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F1A, F1, F10%, F1wb6.5, F1wb and F1wob of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

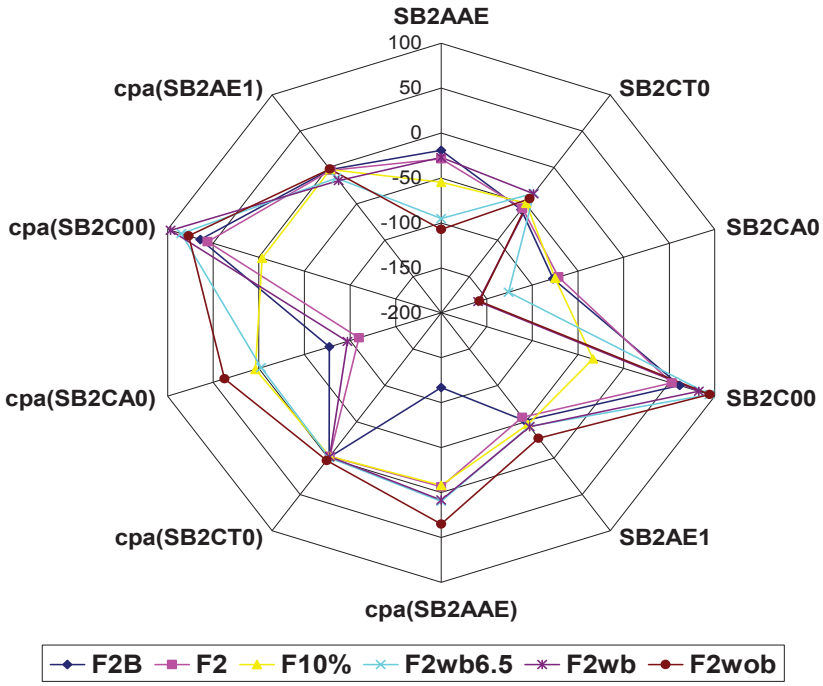

Figure 55: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F2B, F2, F10%, F2wb6.5, F2wb and F2wob of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

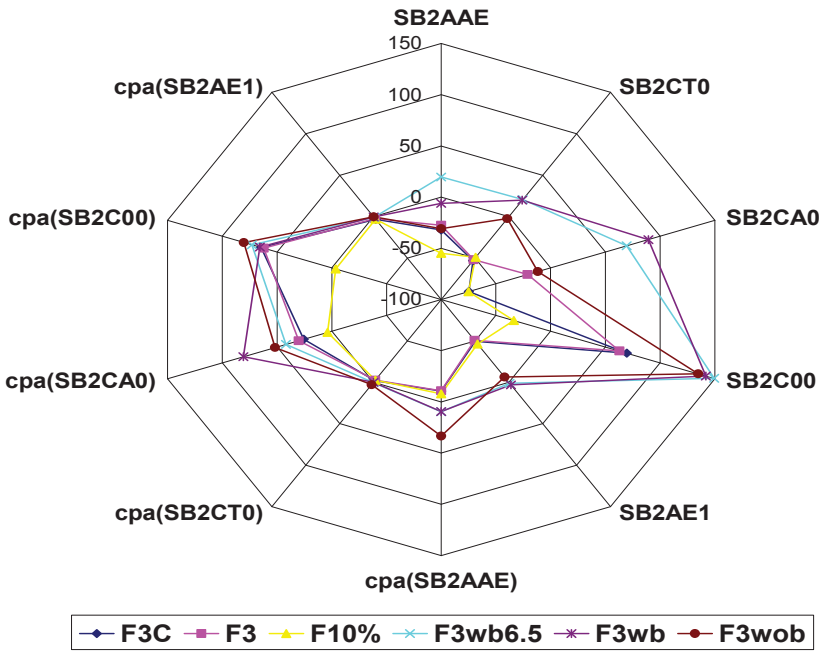

Figure 56: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F3C, F3, F10%, F3wb6.5, F3wb and F3wob of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

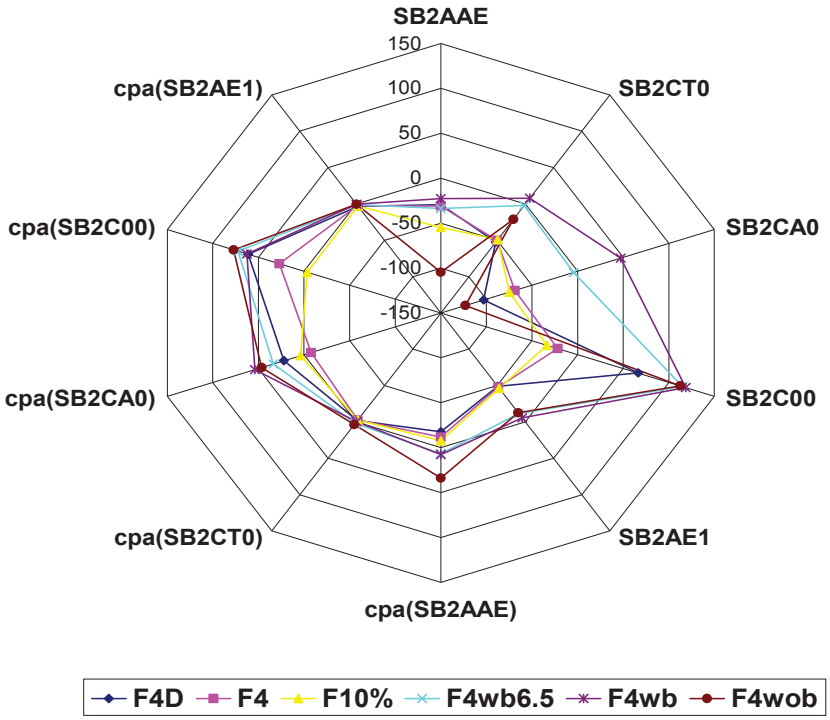

Figure 57: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F4D, F4, F10%, F4wb6.5, F4wb andF4wob of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

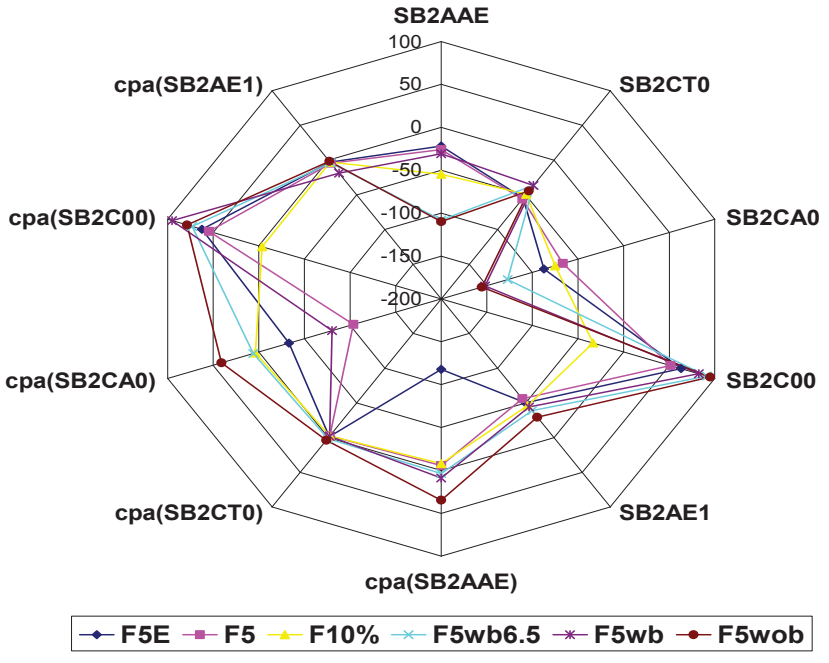

Figure 58: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F5E, F5, F10%, F5wb6.5, F5wb and F5wob of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

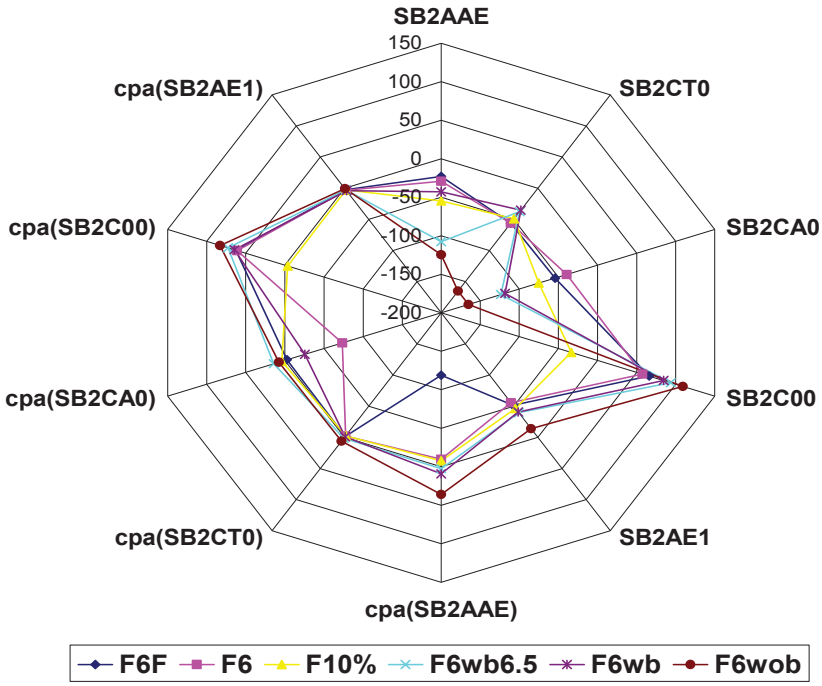

Figure 59: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F6F, F6, F10%, F6wb6.5, F6wb and F6wob of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

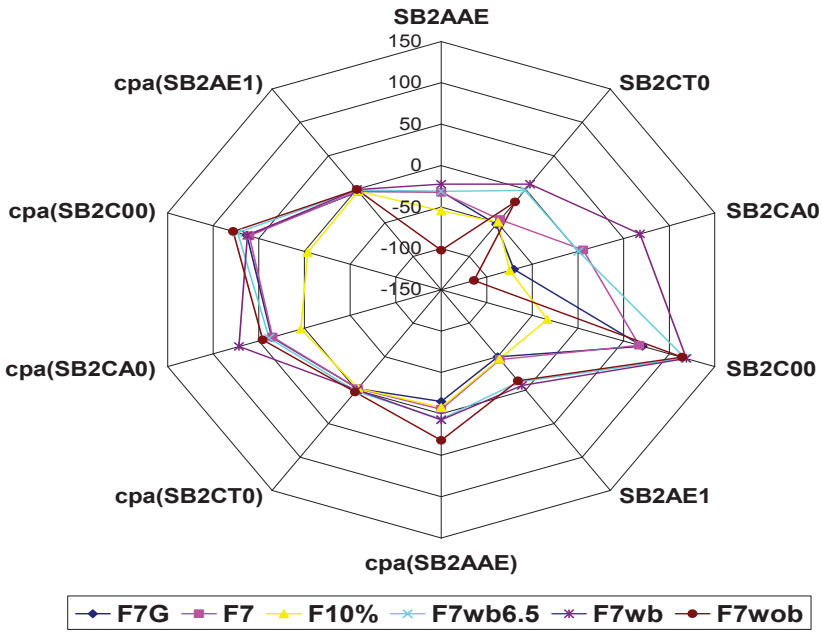

Figure 60: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F7G, F7, F10%, F7wb6.5, F7wb and F7wob of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

The sensor signals obtained for those five types of formulations were different from each other, this was because the effect of their different pH and ionic strength.
The pH and ionic strength of formulations influence the sensor signals obtained from the taste sensing system SA402B sensors, even for sensors that are not attributed to predict saltiness and sourness. From this, it is recommended that the taste measurement of formulations should include the same excipients and drug concentrations at adjusted pH and ionic strength.

3.4 Taste analysis of 10% L-arginine HCL formulations

The purpose of this study is to assess the feasibility for taste masking and comparison of taste intensity during formulation development of five different formulations with 10 % L-arginine HCL, which were differing in the nature of the used exipients analysed by using taste sensing system SA402B and also tested by a human taste panel. The results were compared to each other. Labelling of the five formulations tested with taste sensing system SA402B and human panel taste, table 11, the respective labelling for the tested formulations is displayed as used in the following figures, from the second preformulation study and the long-term stability study. Before the measurement of the different 10% L-arginine HCL formulations a calibration was performed using 6 aqueous L-arginine HCL solutions (concentration ranged from 1 to 15%). These calibration solutions were analysed three times to check the stability and reproducibility of the sensor responses.

3.4.1 Insent taste sensing system

To predict the effect of pH on sensor signals of taste sensing system SA402B for two types of 10% L-arginine HCL solution formulations have different pH for paediatric use with urea cycle disorders. The first type of formulations F1-F5 contains 10% L-arginine HCL, various types of excipients and the pH for those formulations was adjusted to 6.5. The second type of Fa-Fe formulations was the same as the first type but only the pH was different (table 32). The different pH of those two types of formulations influence on the taste information obtained from the taste sensing system SA402B sensors.

By comparing between the formulations to see the effect of pH on sensor signals of those formulations.The sensor signals obtained for those two types of formulations were different from each other, because the effect of their different pH on sensing sensors.

Figures 61 and 62 show sensor signals, which is the result of Insent taste sensing system SA402B analysis applied to sensor outputs for the same two types of formulations of 10% L-arginine HCL solution, but they have different pH. It can see that taste sensing system can discriminate the same type of formulations of 10% L-arginine HCL solution. It is absolutely impossible for humans to distinguish such a difference, so this result suggests that the ability of taste sensing system to distinguish is by far superior to that of humans. This system would be effective in quality control.

Therefore, it can be concluded that the taste of the first type of F1-F5 formulations was relatively better than the taste of the second type of Fa-Fe formulations depending on the taste information obtained from the taste sensing system SA402B sensors. This means that the optimum pH was 6.5 to get better taste of 10% L-arginine HCL formulations for paediatric use with urea cycle disorders (UCDs).

Table 32: Two types of 10% L-arginine HCL solution formulations have different measured pH.

Component	Fa, F1 % w/v	Fb, F2 % w/v	Fc, F3 % w/v	Fd, F4 % w/v	Fe, F5 % w/v
L-arginine HCL	10	10	10	10	10
Domiphenbromide	0.01	0.01	0.01	0.01	0.01
Disodium EDTA	0.1	0.1	0.1	0.1	0.1
Sucralose	0.05	0.05	0.05	0.05	0.05
Cherry syrup	20	-	-	10	-
Honey	-	10	-	5	5
Raspberry syrup	-	-	20	-	10
Banana flavour	0.02	0.02	-	0.02	0.02
Orange flavour	-	-	0.02	-	0.02
Purified water (ml) to	100	100	100	100	100
pH of Fa-Fe notadjusted	4.2	4.7	4.1	4.4	4.3
pH of F1-F5 adjusted to	6.5	6.5	6.5	6.5	6.5

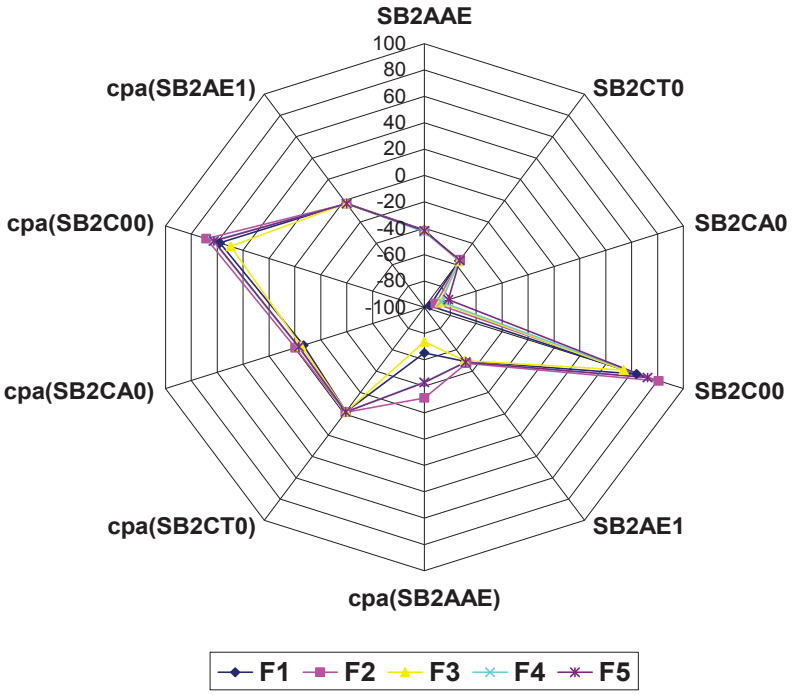

Figure 61: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for F1, F2, F3, F74 and F5 of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

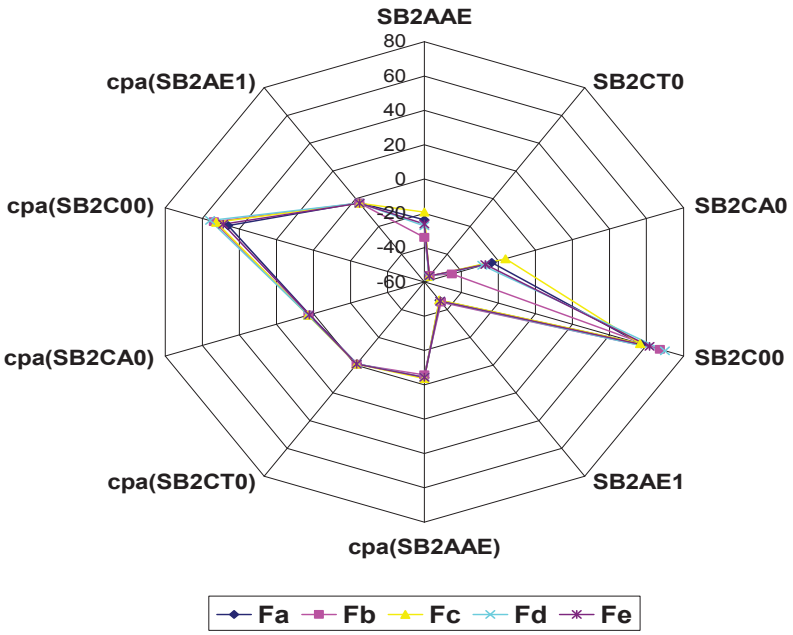

Figure 62: Radar chart of sensor signals of all sensors (SB2AAE, SB2CT0, SB2CA0, SB2C00, and SB2AE1) for three runs, for Fa, Fb, Fc, Fd and Fe of L-arginine HCL. On the link the delayed sensor signals (CPA) are displayed.

3.4.2 Human taste panel

Human taste panel data were collected for the aqueous solution of F1-F5 formulations with 10 % L-arginine HCL. Data are shown in figure 63. The results by human taste panel seem to be roughly matching the results of electronic tongue, indicating a good detection of different L-arginine HCL formulations through the volunteers. The higher deviations can be explained by the high interindividual deviation in taste perception of the volunteers. The formulations F1 and F3 showed higher taste and aftertaste scores than other formulations (p-value < 0.05), indicating a very good taste and masking the umami and bitter taste of L-arginine HCL in F1 and F3. The formulation F2 showed lower taste and aftertaste scores than other formulations (p-value < 0.05), indicating a moderate taste. Therefore, it can be concluded that F1 and F3 were the better formulas than and using cherry and raspberry syrups with other sweetener or flavours was successful to mask the unpleasant taste of L-arginine HCL.

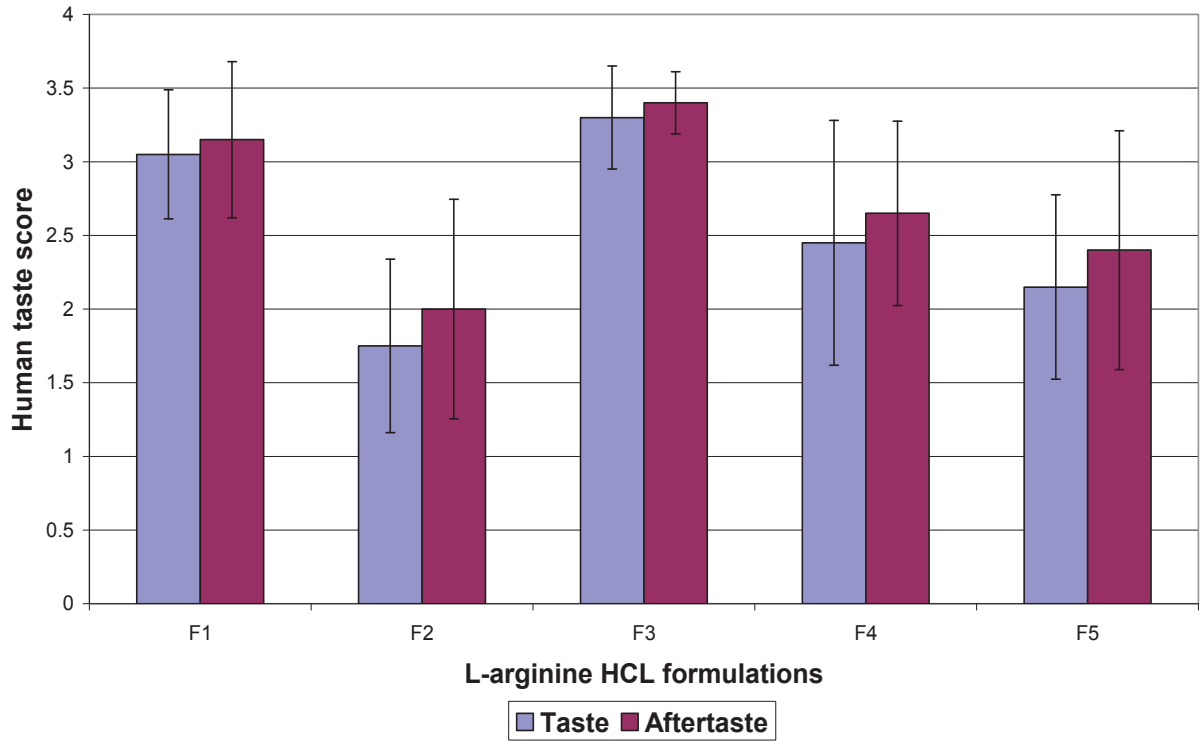

Figure 63: Taste evaluation by adult volunteers of F1-F5 L-arginine HCL formulations on a scale from 0 to 3.5, equalling very bad taste or aftertaste to very good taste or aftertaste (n = 10, mean ± SD).

3.4.3 Correlation taste panel to electronic tongue data

Correlation of electronic tongue to human taste data was achieved using an inverse calibration model based on partial least squares analysis (PLS). In its simplest form, this model specifies the relationship between a single independent variable (taste and aftertaste scores from human taste panel data) with a combination of the multiple components of the electronic tongue sensor data.

Identical samples were analyzed using the electronic tongue. Electronic tongue data were compared to the taste and aftertaste intensity as determined by the human taste panel and a correlation plot was constructed. The electronic tongue data fit the human taste panel data according to the PLS analysis with R^2 = 0.99 (figure 64) for taste and R^2 = 0.98 (figure 65) for aftertaste.

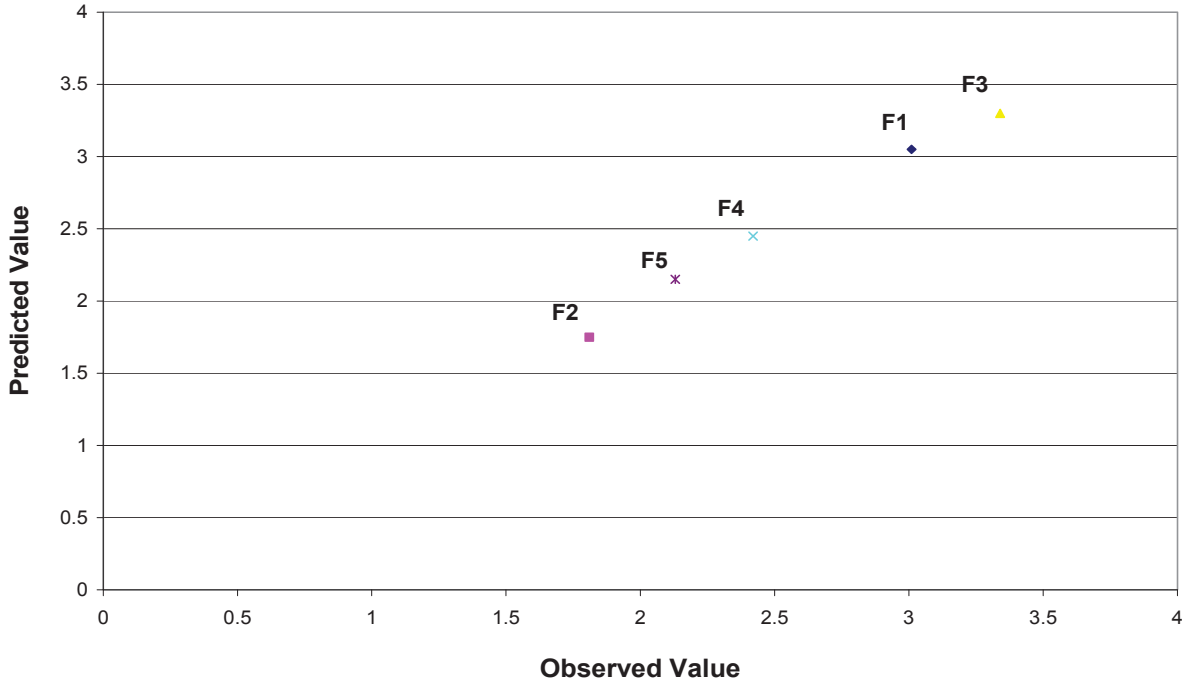

Figure 64: Partial least squares (PLS) regression of human taste panel data vs. electronic tongue data. The *x*-axis is the taste score from the human taste panel data; the y-axis is taste score predicted from the electronic tongue data assuming a correlation exists.

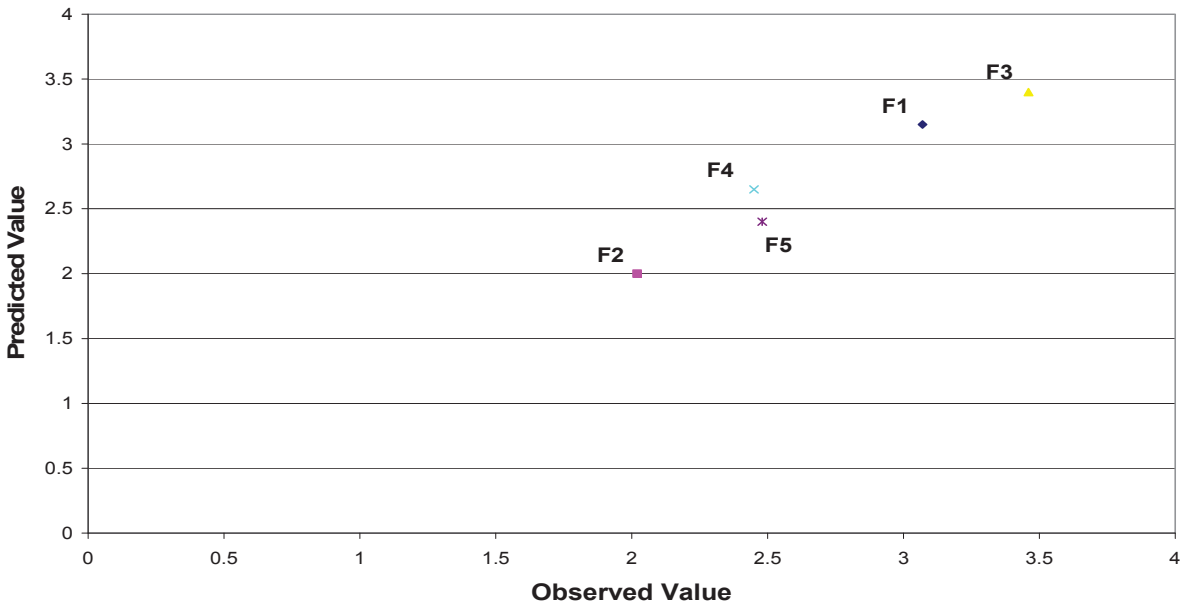

Figure 65: Partial least squares (PLS) regression of human taste panel data vs. electronic tongue data. The *x*-axis is the aftertaste score from the human taste panel data; the y-axis is aftertaste score predicted from the electronic tongue data assuming a correlation exists.

When comparing the PLS plot (figure 64) to the radar chart plot (figure 61), it is important to note that the PLS regression represents all principal components.
More work is needed to understand how the relative positions of samples on the radar chart correspond to the PLS taste and aftertaste scores when the human data are considered.
Electronic tongue taste and aftertaste scores were calculated based on the fit of the data. The calculated taste and aftertaste scores are shown in figures 64 and 65, respectively. The electronic tongue and human scores varied by 0.1 units or less for taste and by 0.2 units or less for aftertaste suggesting that the electronic tongue is able to predict the initial bitterness score of the formulations.

3.5 Conclusion

The taste sensing system SA402B, a multichannel taste sensor system, was evaluated for the feasibility of analysing the taste of L-arginine HCL, an umami and bitter drug, in various formulations containing different kind of excipients. The sensors appear to be sensitive for L-arginine HCL but not to the free base in the range of the tested drug concentrations. They were able to distinguish between different formulations that differed only in the used excipients and not the content of the active. As pharmaceutical taste assessment can demand large panels and elaborate analysis and may raise safety and scheduling concerns, a full taste assessment program can be time-consuming and expensive. Alternatively, the “taste study” may be reduced to an informal gathering of executives, who reach consensus on the best formulation without considering statistical significance or protocol.
Data derived by such a method is highly subjective, limited, and potentially biased. As to the results of all samples tested, the results by human taste panel seem be to roughly match the results of electronic tongue. Overall the taste sensing system SA402B proved to be a suitable tool for evaluating the taste of L-arginine HCL formulations.

It is also suggested that the multichannel taste sensor for the detection of suppression of bitterness by sweet substances and other sensory evaluations of oral dosage forms of bitter drugs with taste inhibitors need to be further investigated for future applications. This would help in the development of more palatable and acceptable dosage forms.

D Summary

In the present investigations the need for child-appropriate drug formulations of L-arginine was detected. It became obvious, that the most prominent need is for a taste masked oral liquid and sustained-release multiparticulate dosage forms. L-arginine is currently used “unlicensed” for treatment of urea cycle disorders in paediatric populations.

The dosage of the L-arginine in dependency of age was elaborated, which enables the comparison of the actual dosage given in the university hospital Düsseldorf with the recommendations given in the literature. In addition to partially considerable differences between different dosage recommendations given in the literature, considerable discrepancies between actual dosage and dosage recommendations could be demonstrated.

The collected drug information was evaluated in comparison with the formulations recommended in the EMEA document “Formulations of choice for the paediatric population” for the different age-groups. Oral liquids are recommended for preterm newborn infants, term newborn infants, infants and toddlers, while multiparticulates are recommended for children (preschool, school) and adolescents.

An urgent need was detected for L-arginine HCL in terms of a new, stable child-appropriate formulation. New oral liquid formulations with L-arginine HCL were developed that meet all requirements for a child-appropriate formulation. The quality of the developed formulations was thoroughly investigated.

The taste masked oral liquid is intended for the use in neonates with urea cycle disorders. In contrast to common oral liquids prepared in hospital or community pharmacies it lowers the risk of incorrect preparation or miscalculation of L-arginine HCL content for newborns, which lead to dosing errors and can be avoided by using ready-to-use formulations.

Five taste masked oral L-arginine HCL 10% solution formulations of high quality were developed and comparatively characterized. The formulations underwent stability testing for 12 months meanwhile the testing of physical and chemical properties were included.

The formulations were stable during the tested time period, since the content of the tested L-arginine HCL was in between 97 % and 103 % of the nominal content for all formulations. Now it will become possible to treat newborns and infants with such an adequate L-arginine HCL formulation.

The sustained-release multiparticulates are intended for children (preschool, school) and adolescent patients who are able to swallow small-sized particles together with food. The developed pellets show the self-coating properties which was most recently discovered and described in the present work for the second time.

It was not successful to obtain L-arginine containing extrudates with microcrystalline cellulose or hypromellose prepared through wet extrusion process, in spite of using the lowest feeding rate of granulating liquid because L-arginine is highly water soluble. Hence, the decision was to obtain sustained-release multiparticulates through solid lipid extrusion/spheronization process by using different lipids as binders.

All developed pellet batches were characterised and compared to each other. It was possible to obtain pellets with suitable batch characteristics such as spherical shape with an aspect ratio of below 1.1 and equivalent diameters of approximately 1.52 mm. Narrow particle size distributions with 10% interval of about 100% are obtained. The manufactured pellets did not show sustained drug release properties, but immediate release profiles were obtained for all batches. This might be due to high water solubility of L-arginine that is rapidly released from the lipid matrices when come into contact with the dissolution medium. Different dissolution media with different pH and ionic strength were used but show no differences between the drug releases at 37°C. This applied to all pellet formulations.

The self-coating principle of lipid pellets, possible through the behaviour of the lipid binder during the extrusion/spheronization process, offers interesting new aspects for the use in not only paediatric oral dosage forms.

However, it was not possible to obtain sustained-release dosage forms from the lipid pellets, but immediate release dosage forms. At least taste-masking could be achieved. For a multiparticulate dosage form with sustained-release characteristics there is a need for an additional coating step with a water insoluble polymer.

The taste assessment was performed in vivo using a human taste panel test with adult volunteers and in vitro using a novel chemometric sensor system called “electronic tongue” (Insent taste sensing system SA402B). The electronic tongue was evaluated for the prediction of taste. The Insent system was able to detect differences and to distinguish between different formulations that differed only in the used excipients and not the content of the active. Correlations between taste values from the human taste panel and data obtained from the Insent electronic tongue were possible for taste and aftertaste. Electronic tongues offer valuable alternatives to the common method of human taste panel tests for the evaluation of taste, especially in cases where taste assessment studies are difficult, as in the formulation development for paediatric patients due to regulatory, ethical or organisational issues.

E Zusammenfassung

Die vorliegende Untersuchung befasst sich mit der Entwicklung von kindgerechten, geschmacksmaskierten L-Argininformulierungen. Es wurde offensichtlich, dass ein großer Bedarf an peroralen Flüssigkeiten mit geschmacksmaskiertem L-Arginin sowie multipartikulären Darreichungsformen mit verlängerter Wirkstofffreisetzung besteht. L-Arginin wird derzeit zur Behandlung von Erkrankungen des Harnstoffzyklus bei pädiatrischen Patientengruppen im “unlicensed use” verwendet.
Die Dosierung des L-Arginins wurde in Abhängigkeit vom Alter herausgearbeitet, wobei die tatsächliche Dosierung im Universitätskrankenhaus Düsseldorf mit den Empfehlungen in der Literatur verglichen wurde. Zusätzlich zu den in der Literatur gefundenen, teilweise erheblichen Unterschieden in den Dosierungsmpfehlungen konnten erhebliche Abweichungen zwischen der tatsächlichen Dosierung und den Empfehlungen aufgezeigt werden.
Die Dosierungsangaben wurden mit den Empfehlungen in dem EMEA-Dokument “Formulations of choice for the paediatric population” für die verschiedenen Altersgruppen verglichen. Während perorale Flüssigkeiten hauptsächlich bei Frühgeborenen, Neugeborenen, Säuglingen und Kleinkindern empfohlen werden, sind multipartikuläre Arzneiformen bei Vorschulkindern, Schulkindern und Jugendlichen vorzuziehen.

Eine stabile kindgerechte Arzneizubereitung für L-Arginin HCL wurde für dringend notwendig erachtet. Deshalb wurden flüssige perorale Zubereitungen mit L-Arginin HCL entwickelt, die alle Anforderungen einer pädiatrischen Formulierung erfüllen. Die Qualität der entwickelten Arzneizubereitungen wurde eingehend untersucht.
Die geschmacksmaskierte perorale Flüssigkeit ist für den Einsatz bei Neugeborenen mit Defekten des Harnstoffzyklus vorgesehen. Im Gegensatz zu bisherigen peroralen Flüssigkeiten, die in Krankenhausapotheken oder öffentlichen Apotheken hergestellt werden, soll das Risiko falscher oder fehlerhafter L-Arginin-Zubereitungen für Neugeborene durch gebrauchsfertige Formulierungen vermieden werden.

Fünf geschmacksmaskierte perorale Lösungen mit jeweils 10% L-Arginin wurden entwickelt und vergleichend charakterisiert. Die Formulierungen wurden eingelagert und für 12 Monate einer Stabilitätstestung unterzogen, die physikalische und chemische Prüfungen umfasste. Die Formulierungen waren im geprüften Zeitraum stabil, der Gehalt an L-Arginin HCL betrug bei allen Formulierungen innerhalb des betrachteten Zeitraumes zwischen 97% und 103% des Sollgehalts. Durch die Untersuchungen wird es möglich, die Behandlung von Neugeborenen und Säuglingen mit einer kindgerechten L-Arginin HCL-Formulierung durchzuführen.
Multipartikuläre Darreichungsformen mit verlängerter Wirkstofffreisetzung sind für Vorschulkinder, Schulkinder und Jugendliche vorzuziehen, die in der Lage sind, kleine Teilchen zusammen mit Lebensmitteln zu schlucken.

L-Arginin-Extrudate konnten nicht mittels Feuchtextrusion hergestellt werden, da L-Arginin wegen seiner hohen Wasserlöslichkeit trotz niedrigster Förderrate an Granulationsflüssigkeit nicht verarbeitet werden konnte. Daher wurden für die multipartikulären Arzneiträger zwei verschiedene Lipide als Bindemittel zur Extrusion/Sphäronisation eingesetzt. Die entwickelten Pellets zeigten eine „self-coating“-Beschichtung, die in der Literatur bisher erst einmal beschrieben wurde. Diese Beschichtung bei Lipid-Pellets, die durch das Verhalten der Lipid-Binder bei der Extrusion/Sphäronisation ermöglicht wird, bietet interessante neue Aspekte für den Einsatz von oralen multipartikulären Darreichungsformen, nicht nur bei Kindern.

Verschiedene Pellet-Chargen wurden hergestellt und hinsichtlich ihrer physikalischen Eigenschaften verglichen. Es war möglich, Pellets mit sphärischer Form mit einem orthogonalem Seitenverhältnis von weniger als 1.1 und einem Durchmesser von ca. 1,52 mm herzustellen. Es konnte eine enge Korngrößenverteilung von annähernd 100% im 10%-Intervall erzielt werden. Trotz der Selbst-Beschichtung der Pellets konnte allerdings keine verlängerte Freisetzung des L-Arginins aus den Lipid-Pellets erreicht werden, sondern nur eine verzögerte oder eine schnelle Freisetzung. Trotz der Verwendung unterschiedlicher Freisetzungsmedien mit verschiedenen pH-Werten und Ionenstärken wurden keine Unterschiede in den Dissolutionsprofilen bei 37°C festgestellt. Dies gilt für alle Chargen der Pelletformulierungen.

Bei gut wasserlöslichen Arzneistoffen sind die Möglichkeiten von Lipidpellets für eine verlängerte Freisetzung offenbar beschränkt. Eine Geschmacksmaskierung des Arginins ist hingegen durchaus möglich.
Für eine multipartikuläre Arzneiform mit verlängerter Freisetzung ist es notwendig, eine zusätzliche Beschichtung mit einem wasserunlöslichen Polymer vorzunehmen.

Die Geschmacksbewertung der entwickelten Zubereitungen von L-Arginin fand sowohl durch erwachsene Probanden, als auch unter Verwendung eines neuartigen Chemometrie-Sensor-Systems statt, das auch als "elektronische Zunge" bezeichnet wird (Insent SA402B). Das Insent-System war in der Lage, zwischen den einzelnen Formulierungen zu unterscheiden, obwohl sie sich nur in den verwendeten Hilfsstoffen und nicht im Gehalt der aktiven Komponente unterschieden. Korrelationen zwischen der Geschmacksbewertung der Probanden und den Daten der elektronischen Zunge waren sowohl für den Geschmack wie auch den Nachgeschmack möglich.
Elektronische Zungen bieten eine neue wertvolle Alternative zu der bisher üblichen Methode der menschlichen Geschmackstests, vor allem in Fällen, in denen die Geschmacksuntersuchungen wegen gesetzlicher, organisatorischer oder ethischer Probleme, wie zum Beispiel bei pädiatrischen Patienten, schwierig sind.

F Experimental Part

1. Materials

1.1 L-arginine

The used L-arginine qualities are displayed in table 33.

Table 33: L-arginine qualities

L-arginine	Ph. Eur.	07H16-N01	Fagron GmbH Co.KG, Barsbüttel, Germany.
L-arginine HCL	Ph. Eur.	07J16-N13	Fagron GmbH Co.KG, Barsbüttel, Germany.

1.1.1 Characterization of L-arginine

There are no chemical reference standards for L-arginine or L-arginine HCL available worldwide. Therefore, it was required to characterize the used starting material as APIs by various analytical techniques. Hence, impurities and degradation products can be identified and quantified. The results from these studies can be used for a dossier on the pharmaceutical quality of the API sources. L-arginine as used in the present studies is a white or almost white crystalline powder, obtained as practically odourless crystals.

1.1.1.1 Physical properties

1.1.1.1.1 X-Ray Powder Diffraction Pattern

To evaluate the crystalline structures of the L-arginine, qualitative x-ray diffraction measurements performed by the Miniflex apparatus (Rigaku, Tokyo, Japan) with powder diffractometer with Bragg-Brentano-geometry was used. Sample preparations into the aluminium frames were performed by mounting the front of the frames on smooth Teflon® plate. A suitable amount of L-arginine was filled into the window. To prevent a preferential orientation of the particles, the samples were compressed with a slide. Diffraction patterns were obtained at a voltage of 30 kV and a current of 10 mA. Scanning was performed in the two-Theta-Scale range from 5° to 70°. The scanning speed was 2 °/min at an intensity of 1000 cps and the distance between two data points 0.02°.The x-ray powder pattern of L-arginine is found in figure 66.

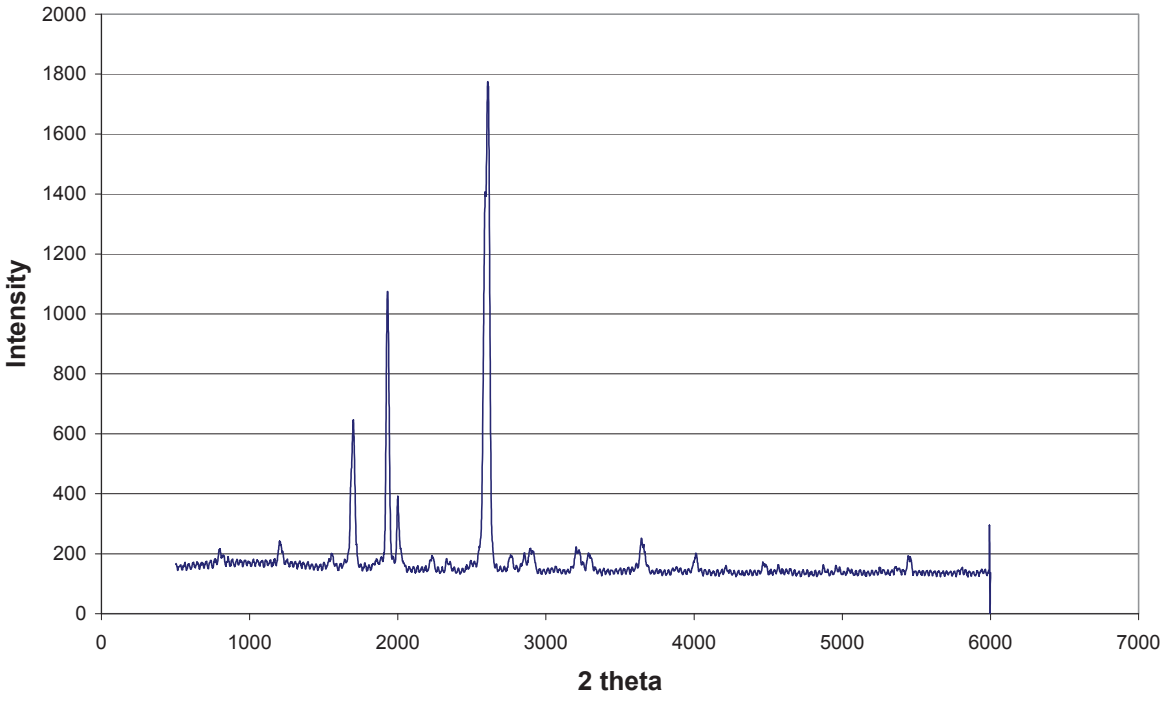

Figure 66: X-ray powder diffraction pattern of commercially obtained L-arginine.

1.1.1.1.2 Particle Morphology

When isolated from water, L-arginine is obtained as minute round crystals (figure 67). A commercial sample was evaluated using optical microscopy, with the data being obtained on a Leica Diastar optical microscopy system (Leica MZ 75, Cambridge, UK).

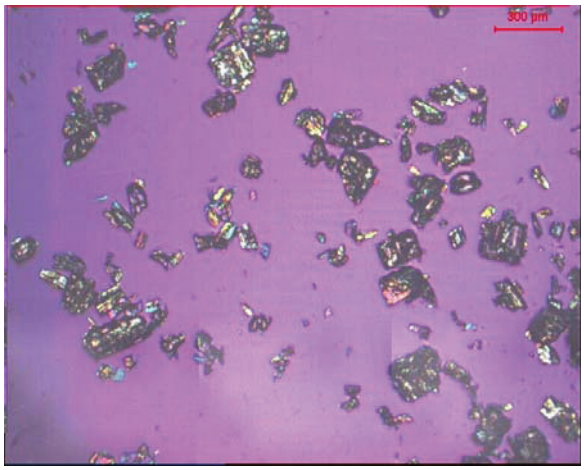

Figure 67: Photomicrograph of commercially obtained L-arginine, obtained at a magnification of 200x.

1.1.1.1.3 Thermal Methods of Analysis

1.1.1.1.3.1 Melting Behaviour

L-arginine is observed to melt at 234°C with decomposition.

1.1.1.1.3.2 Differential Scanning Calorimetry

The differential scanning calorimetry thermogram of L-arginine was obtained using a Mettler DSC 821e (Mettler Toledo, Giessen, Germany). The thermogram obtained is shown in figure 68, along with the thermogravimetric analysis. The only detected thermal event was the melting endotherm at 245.07°C, for which the onset temperature was found to be 244.05°C. Integration of the melting endotherm yielded an enthalpy of fusion equal to 94.85 cal/g.

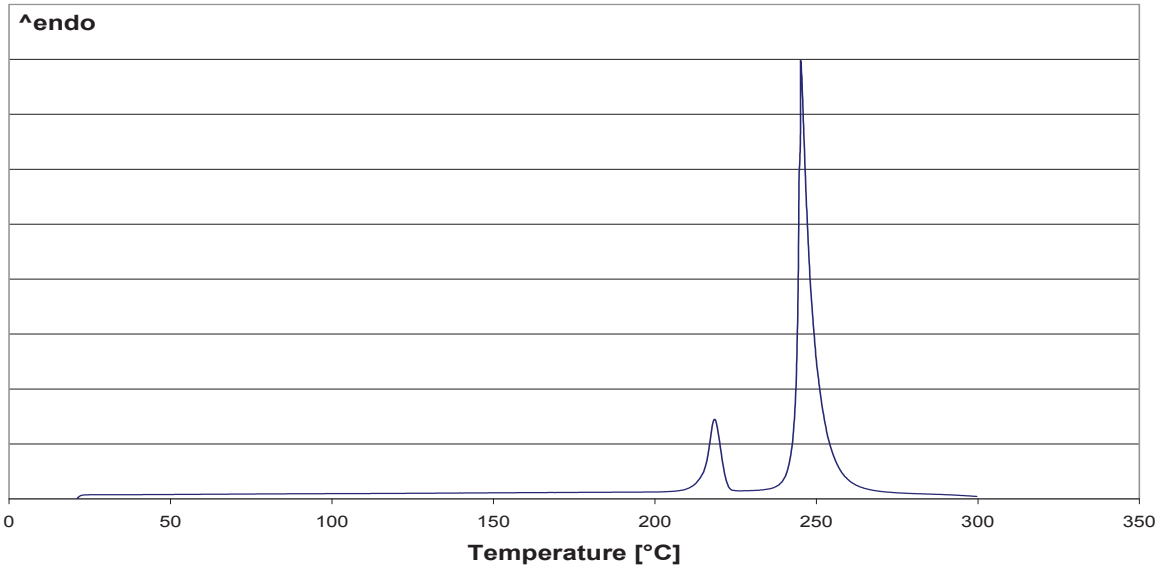

Figure 68: Differential scanning calorimetry of commercially obtained L-arginine.

1.1.1.1.4 Hygroscopicity

L-Arginine is not a hygroscopic substance when exposed to ordinary environmental conditions. The compendial requirement supports this conclusion in that L-arginine dried at 105°C for 3 hours does not lose more than 0.5% of its weight.

1.1.1.1.5 Solubility characteristics

L-Arginine is freely soluble in water (1 g dissolves in 5 mL of water), sparingly or very slightly soluble in alcohol, and practically insoluble in ether.

1.1.1.1.6 Ionization Constants

The ionization constants of L-arginine can be summarized as: pK_{a1} (-COOH) = 2.17, pK_{a2} (α–NH_3) = 9.04 and pK_a (R-group) = 12.48. The isoelectric point of L-arginine is found to be pH= 10.76

1.1.1.1.7 Spectroscopy

1.1.1.1.7.1 Vibrational Spectroscopy

The infrared absorption spectrum of L-arginine was recorded on a Jasco FTIR 300 E spectrometer, using the potassium bromide pellet method. The spectrum scanning 400 to 4000 cm^{-1} is shown in figure 69, and assignments for the observed bands are provided in table 33.

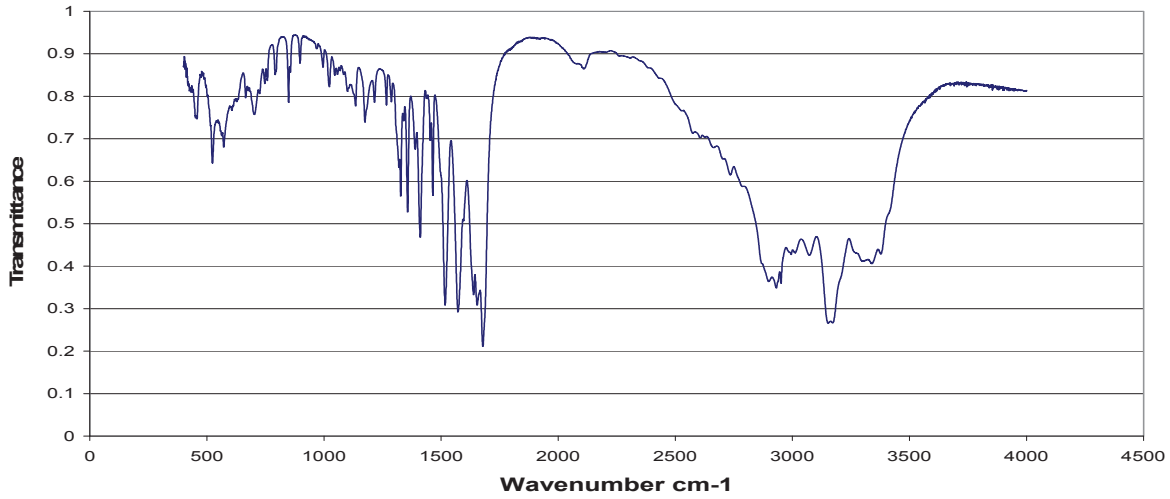

Figure 69: Infrared absorption spectrum of commercially obtained L-arginine, showing the bands in transmission mode.

Table 33: Assignment for the Vibrational Transitions of L-Arginine.

Energy (cm^{-1})	Assignment
3500-2750	O-H stretching mode associated with the hydroxyl groups (intramolecular hydrogen bonding of the carboxylic group)
3200-3100	N-H stretching mode of the amino group and the imine group, overlapped by the strong absorption of the carboxyl O-H group
1710-1690	C=O stretching mode of the carbonyl group
1480	C-H bending mode of the methylene groups
1150-1000	C-O stretch of the carboxylic group

1.1.1.1.7.2 Nuclear Magnetic Resonance Spectrometry

1.1.1.1.7.2.1 ^{1}H-NMR Spectrum

The ^{1}H-NMR spectrum of L-arginine was obtained on a Bruker DRX500 spectrometer operating at 500.13 MHz for ^{1}H, with a 5-mm diameter inverse detection probe and a z-axis gradient coil. The digital resolution was 0.15 Hz per point, (Avance DRX 500, Bruckner, Rheinstetten, Germany) using deuterated water as the solvent and tetramethylsilane as the internal standard. The spectrum is shown in figure 70, and a summary of the assignments for the observed resonance bands is provided in table 34. It should be noted that the protons linked to the nitrogen groups of L-arginine are not observed in the spectra, since they are replaced by deuterium derived from the deuterated water used as the solubilizing solvent.

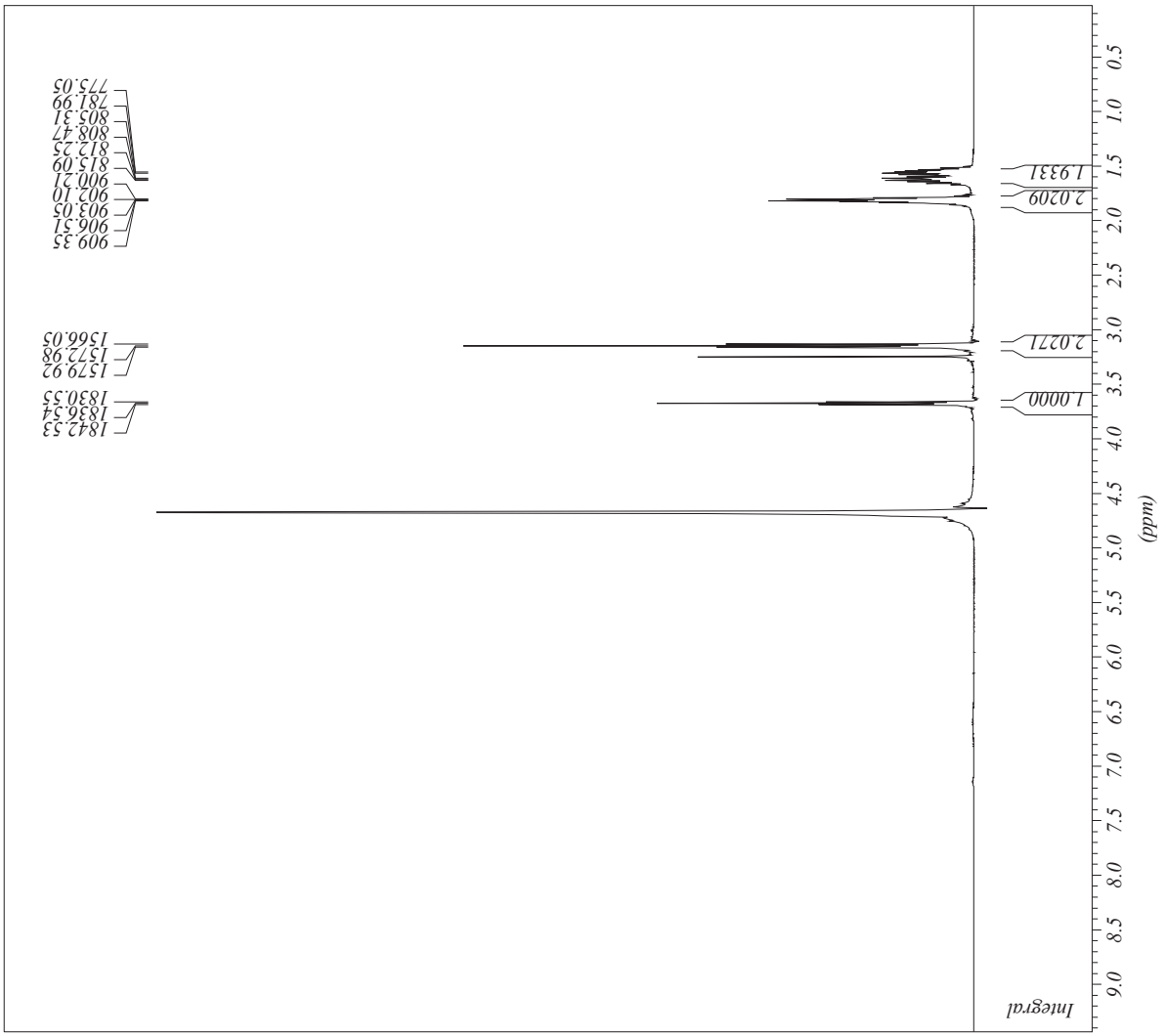

Figure 70: ^{1}H-NMR spectrum of commercially obtained L-arginine.

Table 34: Assignment for the observed ^{1}H-NMR Bands of L-arginine.

Chemical Shift (ppm)	Number of Protons	Assignment
4.75	-	D_2O (H_2O)
3.67	1	Methine proton of the amino acid, Linked to the chiral center at -CH-COOH
3.15	2	Methylene protons of the HN-CH_2 group
1.80-182	2	Protons of CH_2
1.55-1.63	2	Protons of CH_2

1.1.1.1.7.2.2 ^{13}C-NMR Spectrum

The ^{13}C-NMR spectrum of L-arginine was also obtained in deuterated water at ambient temperature, using tetramethylsilane as the internal standard. The one-dimensional spectrum is shown in figure 71, while the Dept-135 spectrum is shown in figure 72. Both spectra were used to develop the correlation between chemical shifts and assignment that are given in table 35.

Figure 71: One-dimensional ^{13}C-NMR spectrum of commercially obtained L-arginine

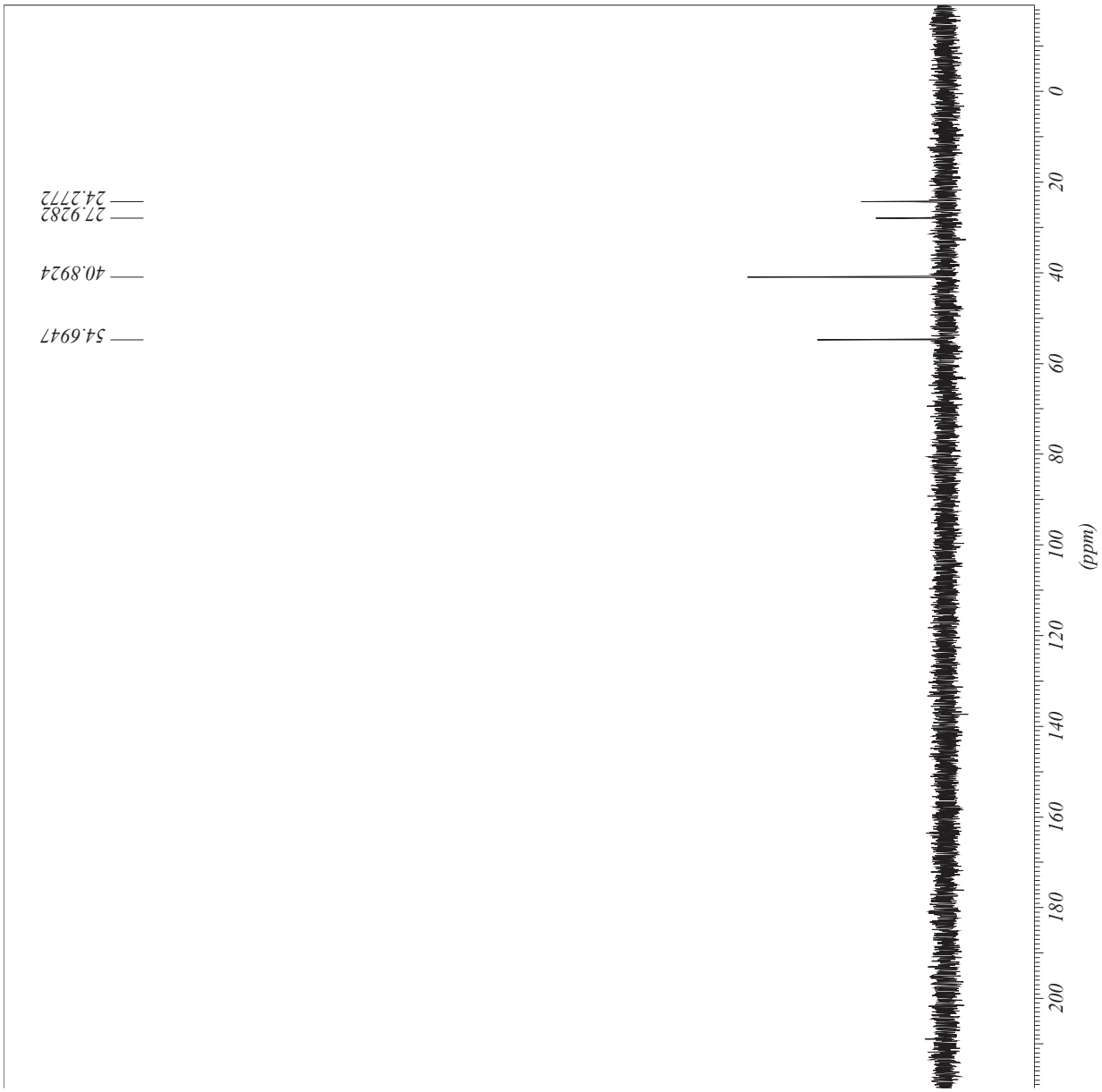

Figure 72: Dept 135 ^{13}C-NMR spectrum of commercially obtained L-arginine.

Table 35: Assignment for the Observed ^{13}C-NMR Bands of L-arginine.

Chemical Shift (ppm)	Assignment (Carbon#)
174.76	1
157.14	6
54.70	2
40.89	5
27.92	3
24.27	4

1.1.1.1.8 Micrometric Properties

1.1.1.1.8.1 Bulk and Tapped Densities

The bulk density of commercially available L-arginine was determined by measuring the volume of known mass of powder that had been passed through a screen into a volume-measuring device, and calculating the bulk density by dividing the mass by the volume. The average bulk density of the L-arginine sample studied was found to be 0.572 g/ml.

The tapped density was obtained by mechanically tapping a measuring cylinder containing a known amount of sample using a tapped density volumeter (StaV, J. Engelsman AG, Ludwigshafen, Germany), after observing the initial volume, the cylinder was mechanically tapped, 100 times over a period of one minute. The tapped density is calculated as the mass divided by the final tapped volume; it was found that the average tapped density of the L-arginine sample was 0.715 g/ml.

1.1.1.1.8.2 Powder Flowability

The Carr Compressibility Index:

$$CI = 100 (V_o – V_f) \quad \text{Eq. (1)}$$

and the Hauser Ratio:

$$HR = V_o / V_f \quad \text{Eq. (2)}$$

are two values that can be used to predict the propensity of a given powder sample to be compressed. The values for V_o (original bulk volume of powder) and V_f (final tapped volume of powder) are obtained during performance of the determination of bulk and tapped density.

The Compressibility Index for L-arginine was found to be approximately 20, indicating that this powdered sample would be 1.24, which also indicates that the powder would exhibit fair degrees of powder flow.

1.1.1.2 Methods of Analysis

1.1.1.2.1 Compendial Tests

1.1.1.2.1.1 European Pharmacopoeia

The EP contains a number of methods that define the compendial article:

		Specification
Identification	Primary: Tests A, C Secondary: B, D, E	A. Complies with specific optical rotation. B. solution is strongly alkaline. C. The Infrared absorption spectrum conforms. D. The principal spot is equivalent to that of the standard in the ninhydrin-positive substance test. E. Yields the expected reaction with β-naphthol and hypochlorite.
Appearance of Solution	General Method (2.2.1)	Solution is clear, and less colored than reference solution BY_6.
Specific Optical Rotation	General Method (2.2.7)	NLT 26.5° and 28.5°
Ninhydrin-Positive Substances	Thin-layer chromatography	Must conform
Chloride	General Method (2.4.4)	NMT 200 ppm
Sulfate	General Method (2.2.13)	NMT 300 ppm
Ammonium	Reaction with litmus paper	NMT 200 ppm
Iron	General Method (2.4.9)	NMT 1 ppm
Heavy Metals	General Method (2.4.8)	NMT 1 ppm
Loss on Drying	General Method (2.2.32)	NMT 0.5% (dried at 100-105°C)
Sulfated Ash	General Method (2.4.14)	NMT 0.1%
Assay	Titration	NLT 98.5% and NMT 101.0% (anhydrous basis)

1.1.1.2.1.2 United states Pharmacopoeia

The USP contains a number of methods that define the compendial article:

		Specification
Identification	General Method <197K>	Must conform
Specific Rotation	General Method <781S>	NLT +26.3° and NMT +27.7° (Test solution: 80 mg/mL, in 6 N hydrochloric acid)
Loss on Drying	General Method <731>	NMT 0.5% (dried at 105°C for 3 hours)
Residue on Ignition	General Method <281>	NMT 0.3%
Chloride	General Method <221>	NMT 0.05% (1.0 g shows no more chloride than corresponds to 0.70 mL of 0.020 N HCl)
Sulfate	General Method <221>	NMT 0.03% (1.0 g shows no more sulphate than corresponds to 0.30 mL of 0.020 N $H_2SO_{4)}$
Iron	General Method <241>	NMT 0.003%
Heavy Metals	General Method <231>, Method I	NMT 0.0015%
Organic Volatile Impurities, Method I <467>	General Method <467>, Method I	Meets the requirements (using water as the solvent)
Assay	Titration	NLT 98.5% and NMT 101.5% (anhydrous basis)

1.1.1.2.2 Elemental Analysis

Elemental analysis of L-arginine, determined in a Carlo Erba EA 1108 Elemental Analyzer, calculated as the average mean of three assays, revealed the following results, table 36.

Table 36: Elemental analysis of L-arginine. The calculated values were obtained using the atomic masses of carbon (12.01), Hydrogen (1.008), nitrogen (14.01) and oxygen (16.00) divided by the molecular weight of L-arginine (M_r174.202).

	%C	%H	%N	%O
Calculated	41.36	8.10	32.16	18.36
Found	41.38	7.29	32.17	18.38

1.1.1.2.3 Titrimetric Analysis

The following procedure has been recommended for the titrimetric analysis of L-arginine. Transfer about 80 mg of L-arginine (accurately weighed) to a 125 mL flask, dissolve in a mixture of 3 mL of formic acid and 50 mL of glacial acetic acid, and titrate with 0.1N perchloric acid VS, determining the endpoint potentiometrically. A blank determination is performed; any necessary corrections are to be made. Each millilitre of 0.1 N $HClO_4$ is equivalent to 8.71 mg of $C_6H_{14}N_4O_2$.

1.2 Lipids quality

Physical properties of two types of lipids were used in the preparation of pellets through an extrusion/spheronization process are displayed in table 37.

Table 37: Used lipid grades.

Features	Precirol® ATO 5 (Glycerol Distearate)	Dynasan® 114 (Glycerol Trimyristate)
Quality	Ph. Eur.	Ph. Eur.
Composition	25-35 % Triglycerides 40-60 % Diglycerides 8-22 % Monoglycerides	95 % < Triglycerides
Melting Point	53-57°C	55-58°C
HLB	2	2
Particle size	30-40 µm	95% <125 µm 2% >250 µm
Hydroxyl number	100	10
Batch number	34196	512158
Supplier	Gattefossé, Weil am Rhein, Germany	Sasol, Witten,Germany

1.3 Other substances

The qualities of other chemical substances were used in this work, table 38.

Table 38: Other used substances.

Substances	Quality	Batch number	Supplier
Domiphenbromide	p.a.	S41264-217	Sigma-Aldrich GmbH, Steinheim, Germany.
Glycerin	USP	K37092393 712	Merck KGaA, Darmstadt, Germany.
Sodium bisulfite	Ph. Eur.	A0240499	ACROS, Geel, Belgium.
Disodium EDTA	Ph. Eur.	10420	Riedel-deHäen, Seelze, Germany.
Trisodium phosphate anhydrous	p.a.	393241	Riedel-deHäen, Seelze, Germany.
Pentane sulfonic acid sodium salt monohydrate	HPLC	A0235749	ACROS, Geel, Belgium.
Sodium dihydrogen phosphate monohydrate	Ph. Eur.	73405	KMF, Lohmar, Germany
Sodium hydroxide	Ph. Eur.	0511903007	J.T.Baker, Deventer, Holland
Cherry syrup		80518108	Caelo, Hilden, Germany.
Honey	Ph. Eur.	70759387	Caelo, Hilden, Germany.
Raspberry syrup		70552117	Caelo, Hilden, Germany.
Sucralose	Ph. Eur.	E0531501	Innosweet, Braunschweig, Germany
Banana flavour		70574137	Caelo, Hilden, Germany
Orange flavour		17175391	NRC, Hamburg, Germany.
Masking flavour TP1000		16795702	NRC, Hamburg, Germany
Masking flavour 501521T		16438908	NRC, Hamburg, Germany
Strawberry flavour		16840003	Firmenich,Kerpen,Germay
Lemon flavour		16993002	Firmenich,Kerpen,Germay
Microcrystalline cellulose (Sanaq 102)	Ph. Eur.	216150	Pharmatrans Sanaq, Basel, Switzerland
Destilled water	Ph. Eur.		in-lab destillation
Hydrochloric acid 1 mol/l (1N)	p.a.		Merck, Darmstadt, Germany
Hypromellose	Ph. Eur.	109650	Syntapharm, Mülheim/Ruhr, Germany
Methanol	HPLC	05Z3677	VWR, Leuven, France
Acetic acid	p.a.	41151235	Carl Roth GmbH, Karlsruhe, Germany
Ethanol	Ph. Eur.	08J280525	VWR, Leuven, France
Phosphoric acid	Ph. Eur.	05017.3000	Bernd Kraft GmbH, Duisburg, Germany
Hydrogen peroxide	p.a.	K23438809845	Merk KGaA, Darmstadt, Germany
Deuterium water	NMR	267781	Euriso-Top, Gif sur Yvette, France
Potassium dihydrogen phosphate	p.a.	18786828	Carl Roth, Karlsruhe, Germany

2. Methods

2.1 Solution preparation

2.1.1 Manufacturing

The aqueous solutions of the API was prepared as follows, L-arginine HCL was dissolved in part of the purified water. Ingredients as listed in each formulation were then added and mixed with a magnetic stirrer. The total volume and the pH were then adjusted. Three bottles of each formulation were prepared.

2.1.2 Storage stability

Three bottles of each formulation were prepared and kept in amber glass bottles (n=3), stored in a drying oven (ET 6130, Heraeus, Hanau, Germany) at 5 °C, 25 °C, 40 °C for 35 days in the first and second preformulation study and for 12 months in the long-term stability study.

2.2 Solution characterisation

2.2.1 HPLC-UV (DAD)

HPLC analysis was performed with a system comprising a solvent pump (Hewlett Packard 1090 Series II (Agilent, Böblingen, Germany). The HPLC with an autosampler injector (Rheodyne, model 7125, Alsbach, Germany) equipped with a 20 µl loop and a diode array spectrophotometer. The mobile phase was prepared as follows: Initially, one litre of pH 3.3 buffer solution was prepared by weighing 0.9 g of 1-pentanesulfonic acid sodium salt, monohydrate and 3.5 g of sodium phosphate monobasic, monohydrate into a suitable container. The pH was adjusted to 3.3 by the addition of phosphoric acid. Subsequently, 850 ml of the pH 3.3 buffer was combined with 150 ml of methanol into a suitable container and mixed. Finally the mixture was degassed before use. The HPLC was conducted using a stationary phase ODS Hypersil C18 5 µm, 250 x 4.0 mm reversed phase column, detecting using UV at 210 µm [45]. The column temperature was set to 40°C. Generally, the run time was 10 minutes, the injection volume was 20 µL, the flow rate was 0.4 mL/min and the mobile phase was pH 3.3 buffer/methanol (85/15, v/v), prepared as described above.

Chromatograms were recorded with an integrator (Hewlett Packard 3396 A Integrator manual Kit, Agilent, Böblingen, Germany). Standard solution of L-arginine HCL was prepared on each day of sample analysis. To calculate the drug concentrations, peak areas were determined and compared to the standard curve, where the standard curve was freshly prepared and used for each analysis. All samples were analyzed with HPLC within a day of each analysis. The retention time of L-arginine monohydrochloride was 8.9 min.

Full validation of the analytical method was required. The establishment of specificity, linearity and limit of quantification is important at the earliest stages, since verification of stability hinges on a suitable method for separating impurities from the active ingredient and at least quantifying the impurities relative to the drug substance. The validation was performed according to ICH Guideline Q2A (validation of analytical methods).The following solutions were prepared and used to determine the precision, accuracy, linearity, limit of detection and limit of quantification and specificity of the HPLC method used.

Standard solution 1 (S1)

About 82.67 mg L-arginine free base, exactly, weighed, are given in a 100 ml volumetric flask and dissolved in 90 ml of distilled water by shacking. Then the solution is filled up to 100 ml.

Test solution (P)

100 mg of L-arginine HCL or 1 ml of 10% L-arginine HCL solution, exactly weighed, are given in a 100 ml volumetric flask and dissolved in 90 ml of distilled water by shacking. Then the solution is filled up to 100 ml. The nominal concentration of the final solution is: 1 mg/ml.

Standard solution 2 (S2)

0.5 ml of test solution P is diluted with distilled water to 100 ml. The nominal concentration of the final solution is: 0.005 mg/ml.

Blind solution (V1)

1 mg domiphenbromide, 10 mg Disodium EDTA, 5 mg sucralose, 1 g cherry syrup, 0.5 g honey, 1 g raspberry, 2 mg Banana flavour, 2 mg Orange flavour and 32.5 mg buffer system are given in a 100 ml volumetric flask and dissolved in 90 ml of distilled water by shacking. Then the solution is filled up to 100 ml.

Blind solution 2 (V2)

To 10 ml of solution V1 distilled water is added and diluted to 100 ml.

The solutions were injected in the following direction, 2 injections of standard solution S1, 1 injection of blind solution V2, 3 injections of test solution P, 1 injection of standard solution S1, 3 injections of standard solution S2 and 1 injection of standard solution S1

The results are only valid if the relative standard deviations of the peak areas of the L-arginine HCL in standard solution S1 don't exceed 2.0%.
For the calculation of the assay of L-arginine HCL the peak areas of the L-arginine HCL corresponding peak are calculated according to equation (3).

$$C\,(Arg.HCL) = \frac{A\,(P) \times e\,(St)}{A\,(S1) \times e\,(P)} \times 1000 \qquad \text{Eq. (3)}$$

C (Arg.HCL) = assay of L-arginine HCL [mg/g]

A (P) = area of L-arginine HCL peak in test solution P

A (S1) = mean area of L-arginine HCL peak in standard solution S1

e (P) = weighed amount of test substance [mg]

e (St) = weighed amount of standard substance [mg]

2.2.1.1 Precision

Following the method described above the standard solution S1 and 6 test solutions P (i.e. P1 to P6) were injected in the following direction:

1 injection of S1, 1 injection of P1, 1 injection of P2, 1 injection of P1, 1 injection of P2, 1 injection of S1, 1 injection of P3, 1 injection of P4, 1 injection of P3, 1 injection of P4, 1 injection of S1, 1 injection of P5, 1 injection of P6, 1 injection of P5, 1 injection of P6, 1 injection of S1.

The results were only valid if the relative standard deviation of the peak areas of the L-arginine HCL in standard solution S1 did not exceed 2.0%.
For the calculation of the assay of L-arginine HCL the peak areas of the L-arginine HCL corresponding peak have been calculated according to Equation (3). Criterion for system suitability (RSD not more than 2.0%) was fulfilled.

The relative standard deviation is calculated as 0.56% for L-arginine and 0.67% for L-arginine HCL, the confidence interval is calculated as 99.69 -100.77%. Hence, the precision of the method is sufficient.

2.2.1.2 Accuracy

The determination of the accuracy is performed using determination of the recovery rate. Following the method described above under 2.2.1. The following solutions were produced in addition:

Stock solution (V3):

About 1 g L-arginine HCL, exactly weighed, was dissolved in 50 ml of distilled water in a 100 ml volumetric flask and diluted to 100 ml with the distilled water. The nominal concentration of the final solution is: 10 mg/ml.

Test solution 50% (P50):

10 ml blind solution V1 and 5.0 ml stock solution V3 were diluted with distilled water a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 0.5 mg/ml.

Test solution 75% (P75):

10 ml blind solution V1 and 7.5 ml stock solution V3 were diluted with distilled water a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 0.75 mg/ml.

Test solution 100% (P100):

10 ml blind solution V1 and 10.0 ml stock solution V3 were diluted with distilled water a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 1 mg/ml.

Test solution 125% (P125):

10 ml blind solution V1 and 12.5 ml stock solution V3 were diluted with distilled water a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 1.25 mg/ml.

Test solution 150% (P150):

10 ml blind solution V1 and 15.0 ml stock solution V3 were diluted with distilled water a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 1.5 mg/ml.

The test solutions and blind solution V2 were injected in a random direction; every solution had to be injected two times. At the beginning two injections of standard solution S1 and then each after 5 injections and at the end one injection S1 was performed. The assay of L-arginine HCL in the test solutions is calculated using equation (4). Criterion for system suitability (RSD not more than 2.0%) was fulfilled.

$$C\ (\%) = \frac{A\ (P) \times e\ (St)}{A\ (S1) \times e\ (P)} \times 100 \qquad \text{Eq. (4)}$$

C (%) = assay of L-arginine HCL [%]

A (P) = area of L-arginine HCL peak in test solutions

A (S1) = mean area of L-arginine HCL peak in standard solution S1

e (P) = weighed amount of test substance [mg]

e (St) = weighed amount of standard substance [mg]

2.2.1.3 Linearity

The linearity is calculated from the results of the tests described under 2.2.1.2. For this purpose from the peak areas the equation of the graph as well as the slope (136244), y-axis-intercept (80536) and correlation coefficient R^2 (0.9996) via linear regression is calculated. It could be demonstrated to be excellent, see figure 73.

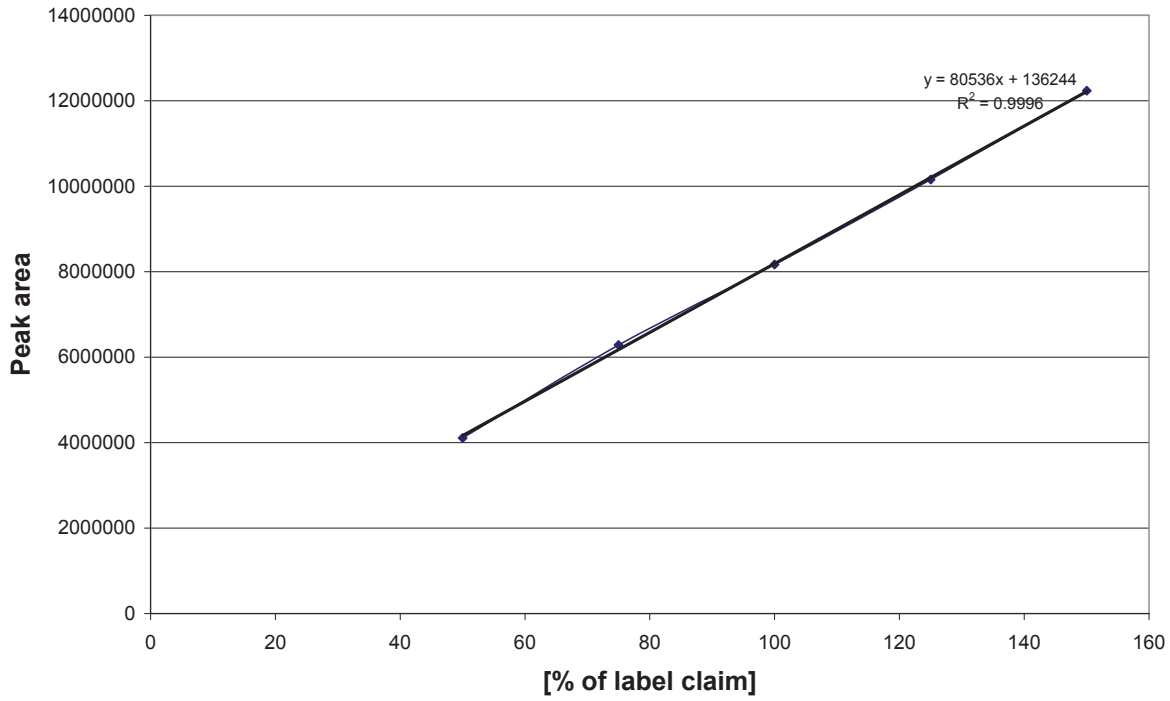

Figure 73: Linearity of L-arginine assay.

2.2.1.4 Limit of detection and limit of quantification

The limit of detection and limit of quantification were determined by preparation solutions of different concentrations of L-arginine HCL and injection of those solutions until the peak height is less than three times of the base line (table 39).

Following the method described above under 2.2.1, the following solutions were produced in addition:

Stock solution (V4):

10 ml stock solution V3 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 1 mg/ml.

Standard solution (S3):

30 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 300 µg/ml.

Standard solution (S4):

20 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 200 µg/ml.

Standard solution (S5):

10 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 100 µg/ml.

Standard solution (S6):

5 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 50 µg/ml.

Standard solution (S7):

2.5 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 25 µg/ml.

Standard solution (S8):

2.5 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 12.5 µg/ml.

Standard solution (S9):

1 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 10 µg/ml.

Standard solution (S10):

0.8 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 8 µg/ml.

Standard solution (S11):

0.4 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 4 µg/ml.

Standard solution (S12):

0.2 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 2 µg/ml.

Standard solution (S13):

0.1 ml stock solution V4 was diluted with distilled water in a 100 ml volumetric flask to 100 ml. The nominal concentration of the final solution is: 1 µg/ml.

Standard solution (S14):

0.1 ml stock solution V4 was diluted with distilled water in a 200 ml volumetric flask to 200 ml. The nominal concentration of the final solution is: 0.5 µg/ml.

Table 39: Limits of detection and quantification of L-arginine.

Solution	Concentration [µg/ml]	
S3	300	Detectable / quantifiable
S4	200	Detectable / quantifiable
S5	100	Detectable / quantifiable
S6	50	Detectable / quantifiable
S7	25	Detectable / quantifiable
S8	12.5	Limit of quantification
S9	10	Limit of detection
S10	8	Limit of detection
S11	4	Limit of detection
S12	2	Limit of detection
S13	1	Not detectable
S14	0.5	Not detectable

The limit of detection was determined as 2 µg/ml and the limit of quantification as 12.5 µg/ml, see table 39. The following chromatogram shows solution S9, which is the limit of detection, figure 74.

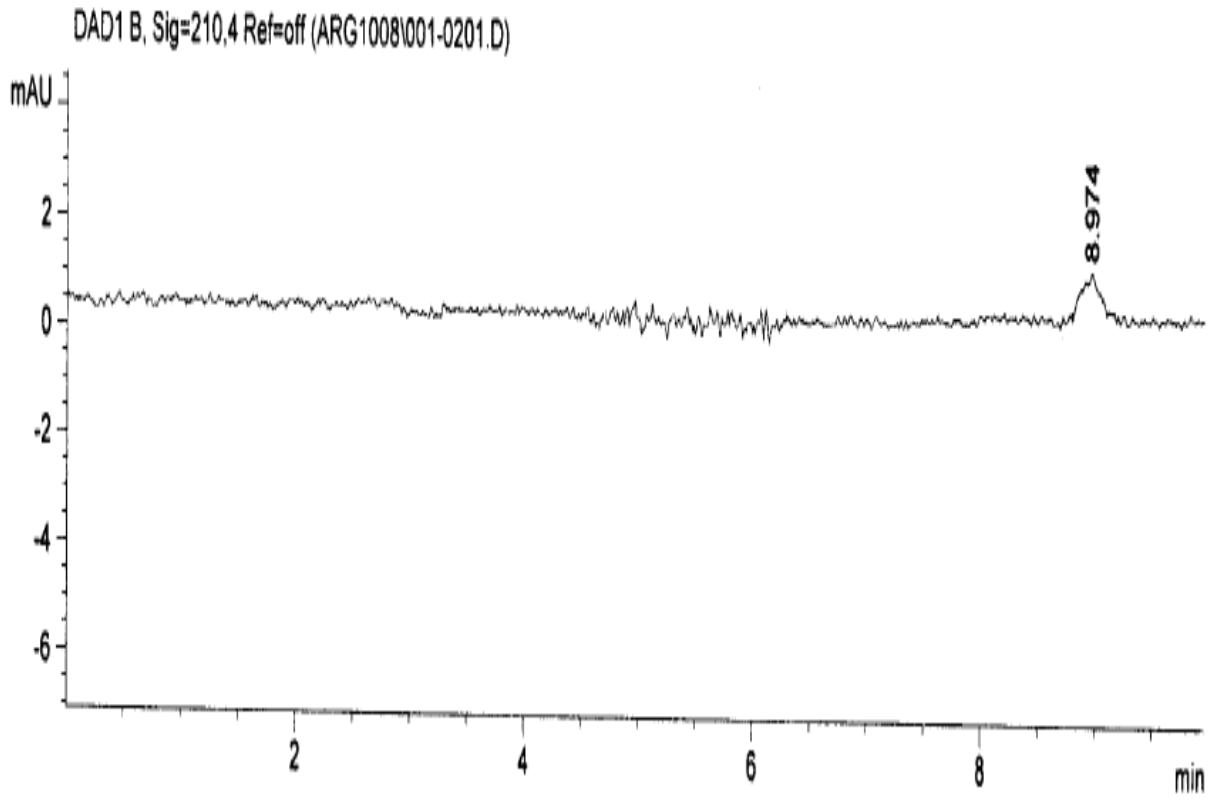

Figure 74: The limit of detection of L-arginine HCL.

2.2.1.5 Specificity

2.2.1.5.1 Chromatograms

Specificity refers to the ability of the method to resolve the species of interest from all other components of the mixture. This is determined by spiking a solution with all the known possible interfering components of the sample. In the case of drug substances and drug products, these would include the synthetic precursors and degradation products. For biological samples, it may be necessary to test the method for potential interferences from any of these interfering species will certainly have an effect on the precision and accuracy of any chromatographic method. To demonstrate the specificity of the method the following solutions were made and injected, mobile phase, solvent (i.e. distilled water), placebo (solution V1), standard solution S1, test solution of substance (P100) and test solution of the formulation, see figures 75, 76, 77, 78, 79, 80, respectively.

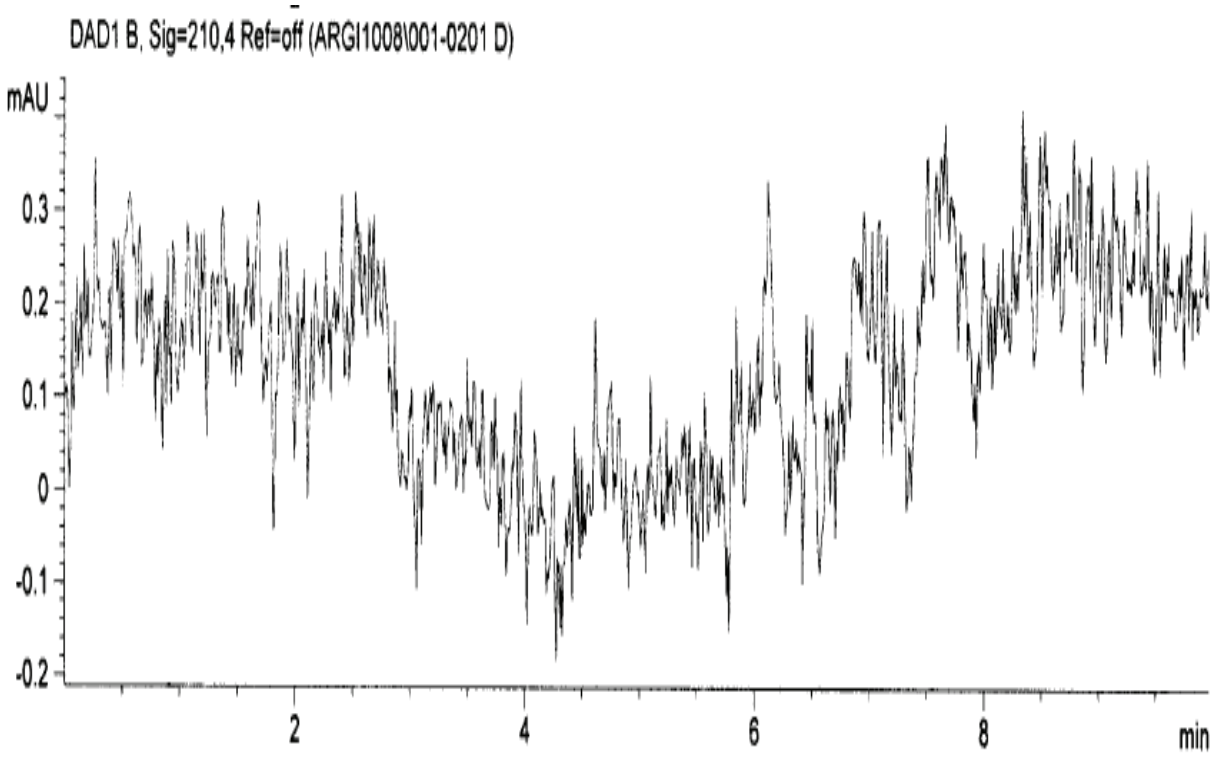

Figure 75: mobile phase chromatogram.

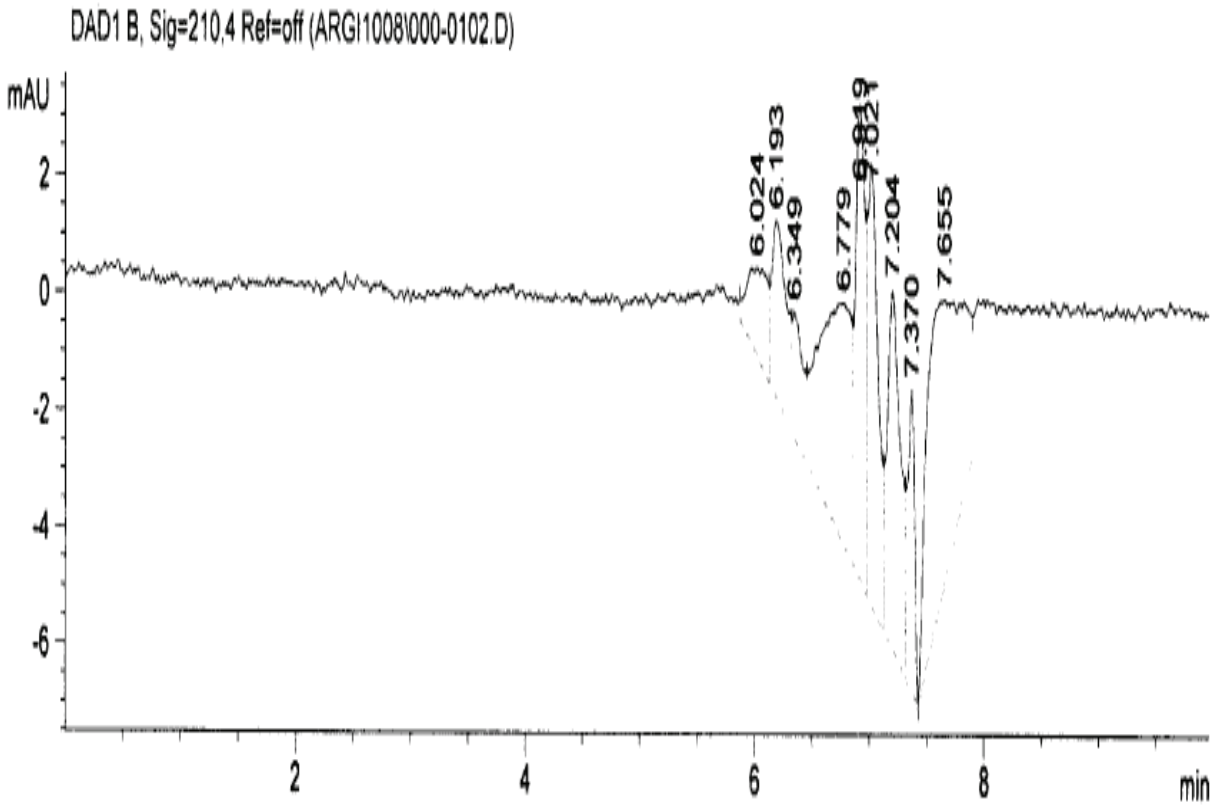

Figure 76: Solvent (i.e. distilled water) chromatogram.

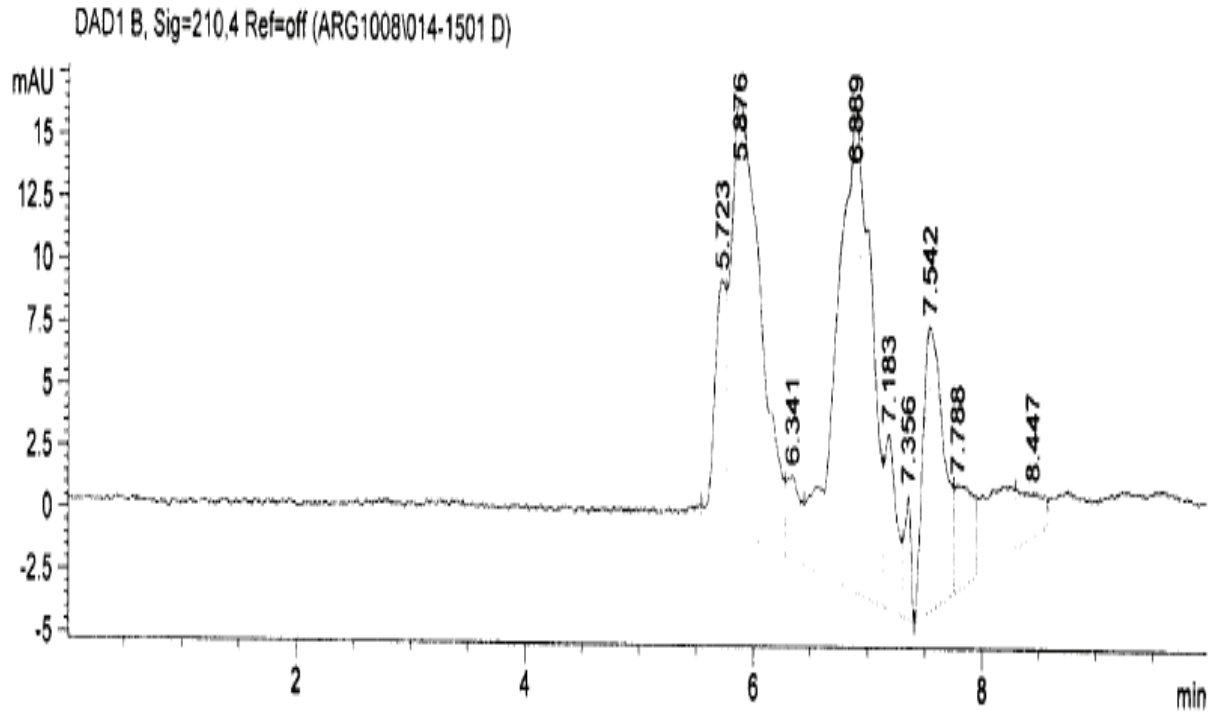

Figure 77: Placebo (solution V1) chromatogram.

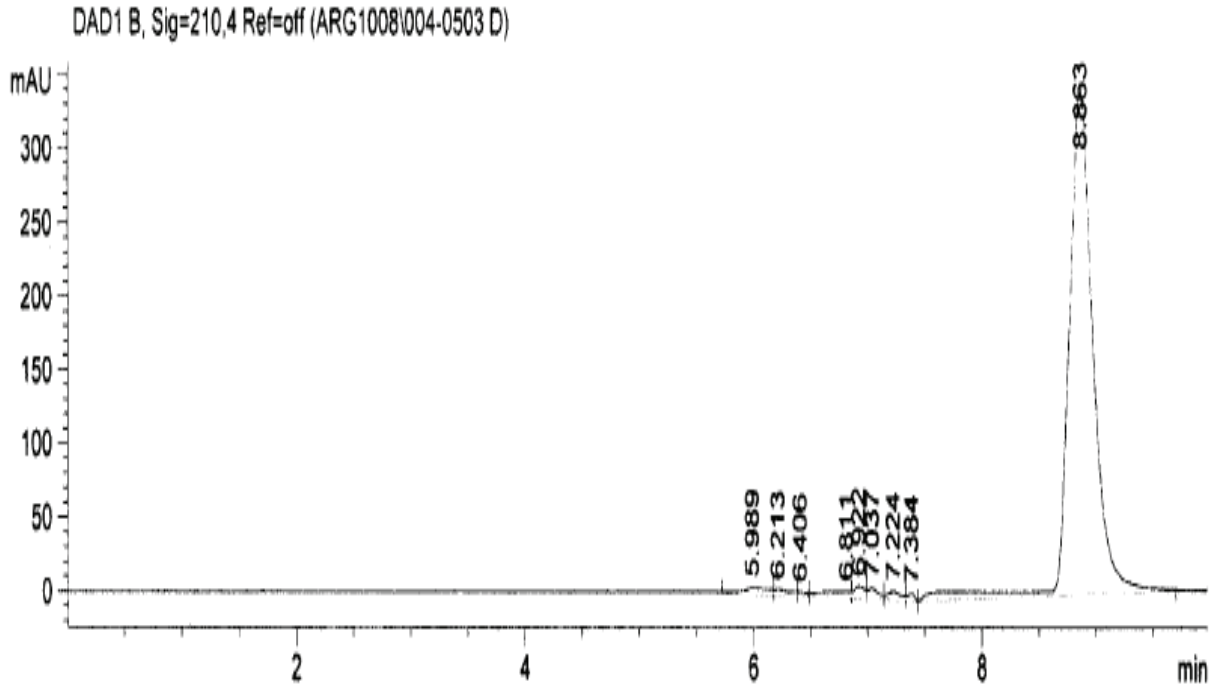

Figure 78: Standard solution S1 chromatogram.

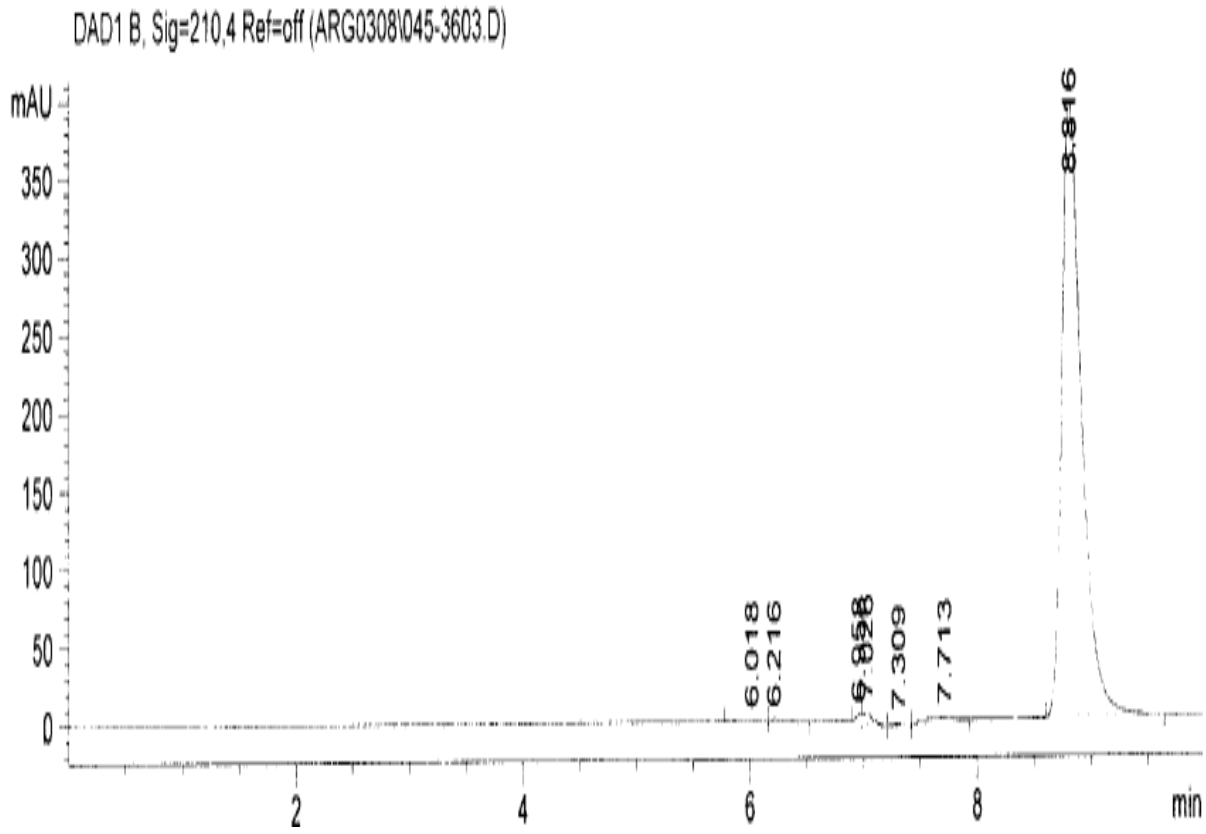

Figure 79: Test solution of substance (P100) chromatogram.

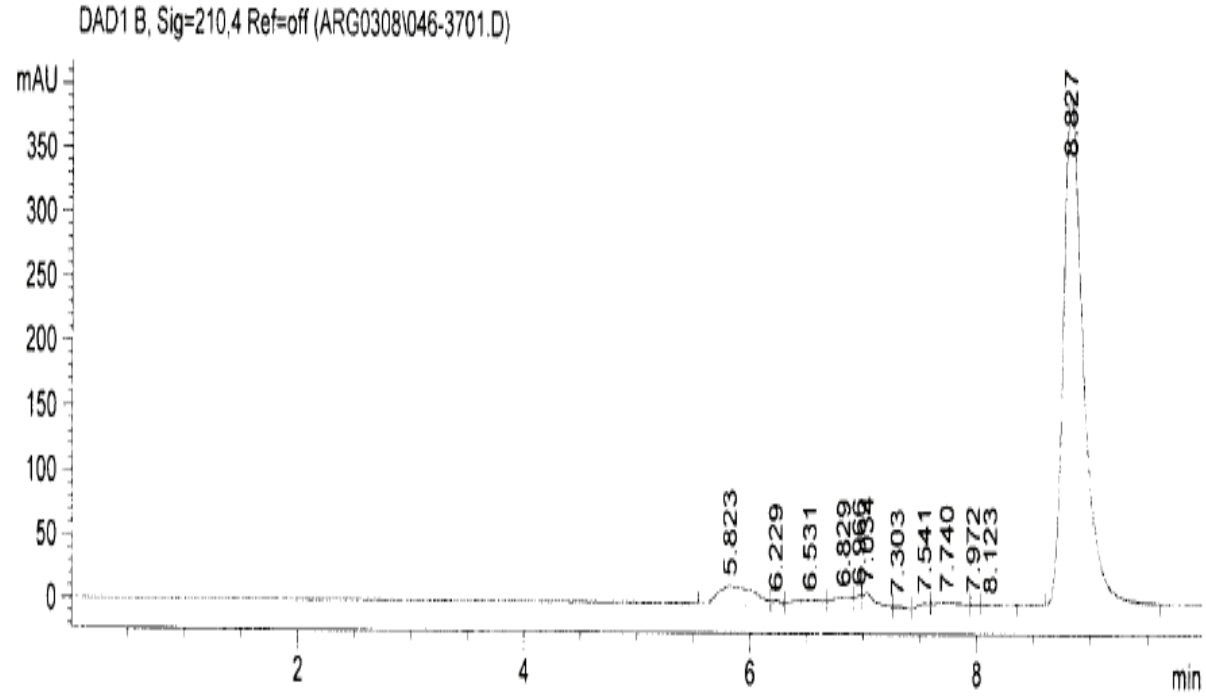

Figure 80: Chromatogram of test solution of the formulation.

2.2.1.5.2 Stress tests

In addition, to demonstrate specificity, L-arginine was treated as follows:

Solution A:

To 2.350 g L-arginine HCL in a 25 ml volumetric flask 2.5 ml ethanol and 20 ml 0.1N hydrochloric acid is added. The flask is kept tightly closed for 72 hours at 80°C in a drying cabinet. After 2 hours for cooling 1 ml solution is neutralised with 1 ml of 0.1N sodium hydroxide and diluted with distilled water to 100 ml, see figure 81.

Solution B:

To 2.350 g L-arginine HCL in a 25 ml volumetric flask 2.5 ml ethanol and 20 ml 0.1N sodium hydroxide is added. The flask is kept tightly closed for 72 hours at 80°C in a drying cabinet. After 2 hours for cooling 1 ml solution is diluted with distilled water to 100 ml, see figure 82.

Solution C:

To 2.350 g L-arginine HCL in a 25 ml volumetric flask 2.5 ml ethanol and 20 ml hydrogen peroxide solution 30% is added. The flask is kept tightly closed for 72 hours at 80°C in a drying cabinet. After 2 hours for cooling to 1 ml solution, 1 ml of 0.1N sodium hydroxide is added and diluted with distilled water to 100 ml, see figure 83.

Solution D:

2.350 g L-arginine HCL is given in a 25 ml volumetric flask. The flask is kept tightly closed for 72 hours at 80°C in a drying cabinet. After 2 hours for cooling the substance is dissolved in distilled water to 100 ml, see figure 84.

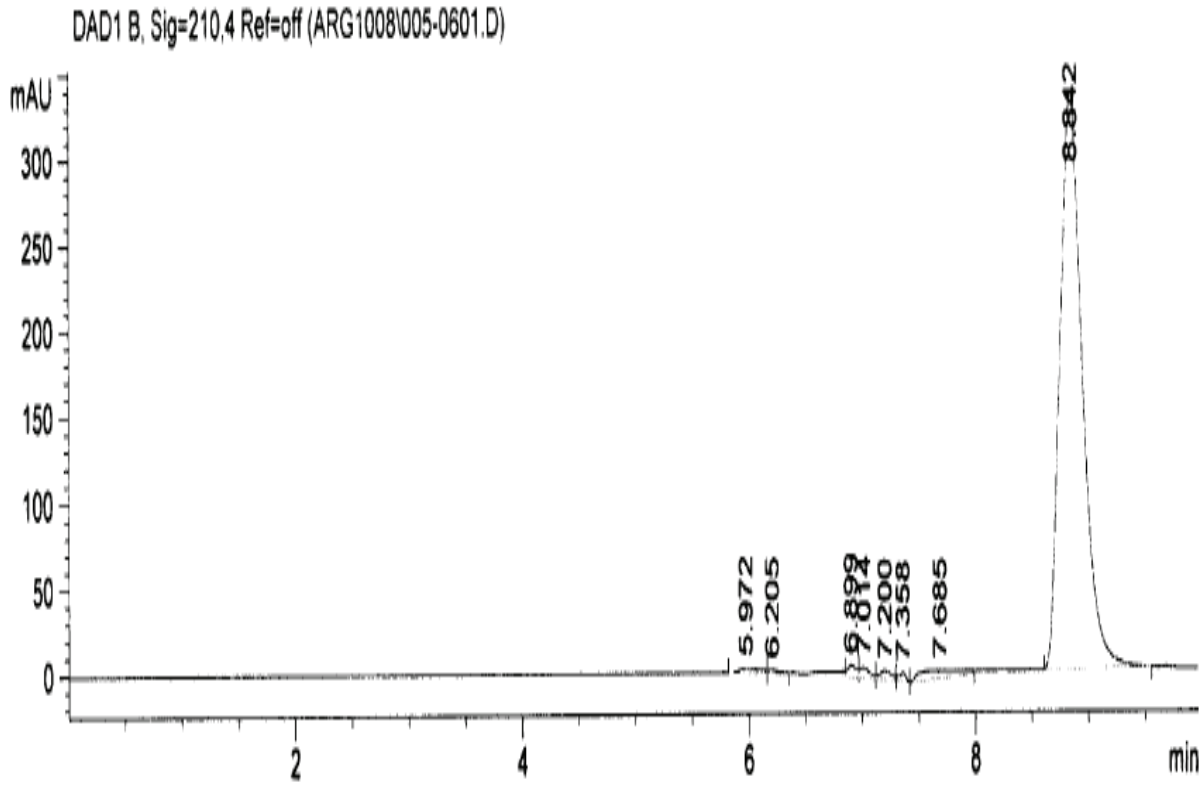

Figure 81: Chromatogram of acid treated L-arginine.

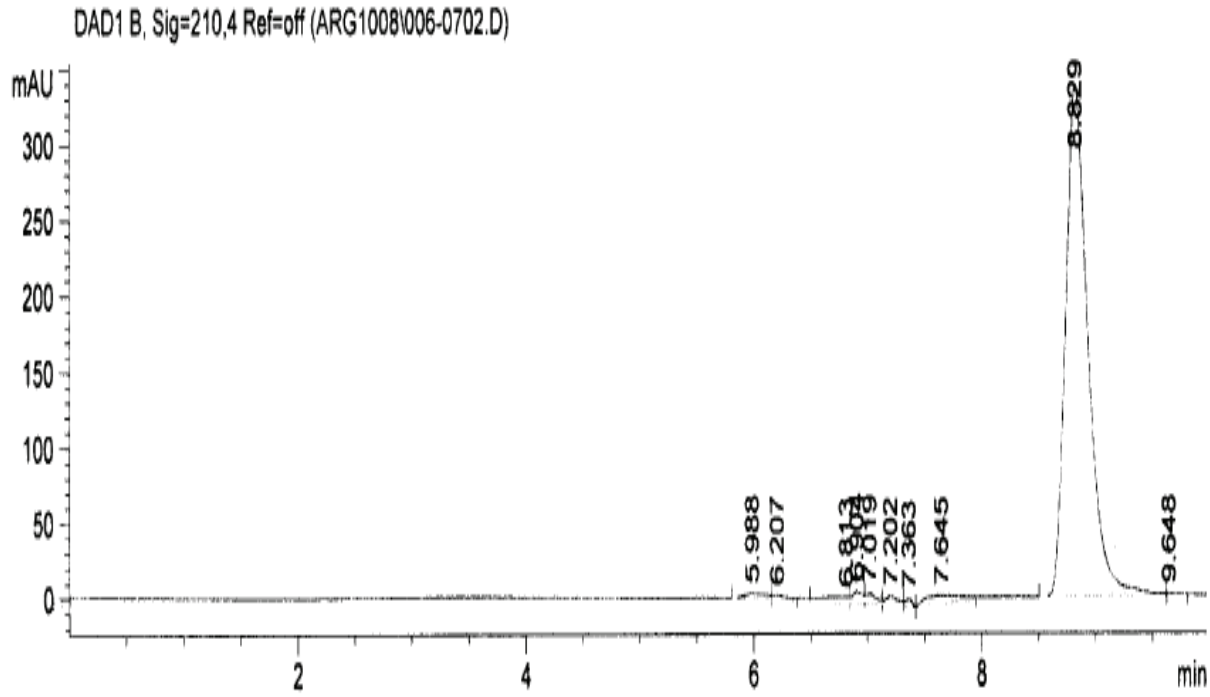

Figure 82: Chromatogram of basic treated L-arginine.

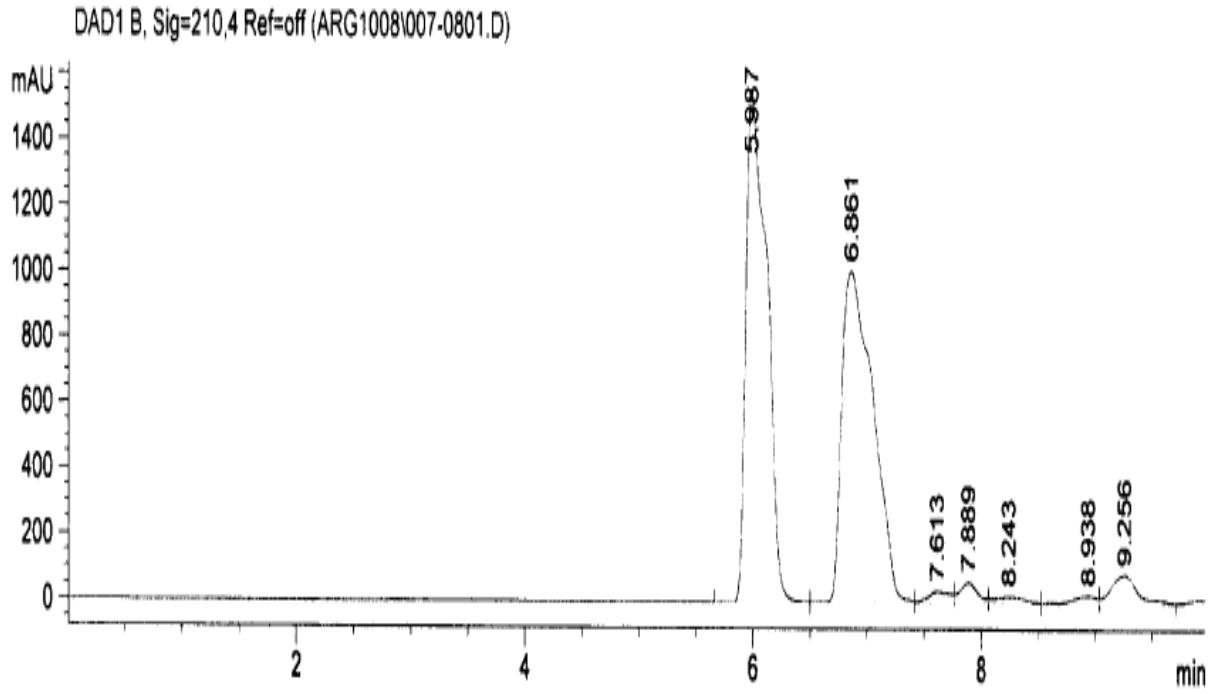

Figure 83: Chromatogram of hydrogen peroxide treated L-arginine.

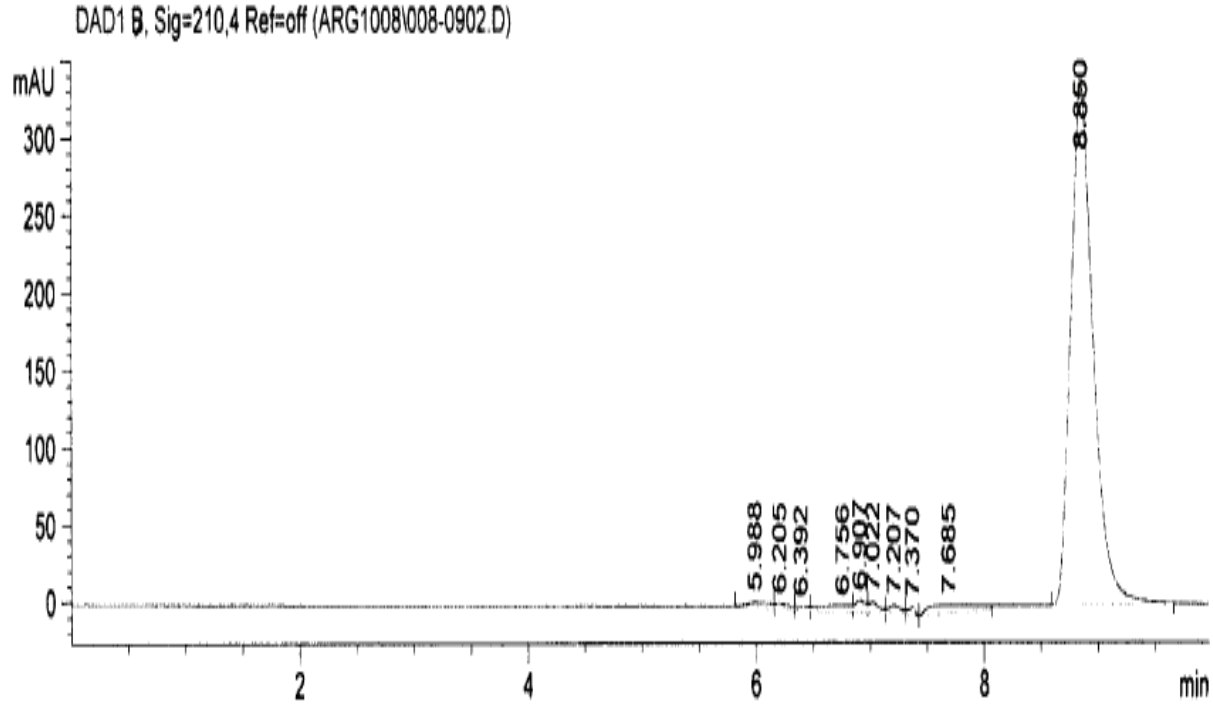

Figure 84: Chromatogram of heat treated L-arginine.

It can be seen from the results of the stress tests that L-arginine was unstable only in the presence of strong oxidizing agent like hydrogen peroxide and fairly stable in the presence of acid, base and at higher temperature in solid state.
The precision was found to be sufficient; the accuracy was determined to be sufficient via the recovery rate which is 100.66%. Linearity could be shown between 50% and 150% of labelled claim of L-arginine. Sufficient limits of detection and quantification were found with concentrations of 2 µg/ml respectively 12.5 µg/ml. The method is specific could be demonstrated, no interaction between mobile phase, placebo and potential degradations are observed.

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2.2.2 Physical and chemical evaluation

The physical and chemical stability testing of L-arginine preparations were performed at day 0, 7, 14, 21, 28 and 35 for the first and second preformulation stability studies and at month 0, 3, 6, 9, 12 for long-term stability study. PH of the samples was measured and the precipitation of the preparations was also visually observed. L-arginine HCL remaining was analysed using a HPLC system (described above).
Samples (1.0 ml) were pipetted, diluted with Distilled water in a 100 ml volumetric flask, and then about 2 ml filtered through 25mm syringe filter W/0.45µm polypropylene membrane. Filtered solution was kept in HPLC vials with screw cap before HPLC assay.

2.2.3 Osmolarity measurement

Osmolality, expressed as mosmol/kg, was measured by freezing-point depression with a micro-osmometer (Knauer, Berlin, Germany). Calibration was performed before every measuring cycle using a 0 mosmol/kg and a 400 mosmol/kg standard solution. The solution (200 µl) was placed in the measuring cap and super cooled by a Peltier device as required for the osmometer. Freezing was initiated by a stir wire. The results could be read off directly in mosmol/kg from the scale. Osmolality was measured on the day the solutions were made.

2.2.4 pH-measurement

The pH-value of the L-arginine HCL formulations was measured with pH-electrodes Calimatic® 766 (knick, Berlin, Germany) and Knick 507 (Berlin, Germany).

2.2.5 Conductivity measurement

The conductivity of the L-arginine HCL formulations was measured with Conductometer E587 (Metrohm, Herisau, Switzerland).

2.2.6 Uniformity of mass of delivered doses from multidose containers

The test according to Ph.Eur. is intended for oral dosage forms such as granules, powders for oral use and liquids for oral use, which are supplied in multidose containers provided at manufacture with a measuring device.
20 doses were weighed individually and taken at random from containers with the measuring device provided and determined the individual and average masses. Not more than 2 of the individual masses deviate from the average mass by more than 10 percent and none deviates by more than 20 percent.

2.2.7 Human Taste Panel

Ten healthy, adult volunteers participated in this trial. Each volunteer randomly tested each sample. Each volunteer was given unlimited amount of time between testing the different samples; bottled distilled water was given in between to rinse the mouth. A scoring system of 0 to 3.5 was employed. A score of 0 indicated very bad taste (unpalatable taste) whereas a score of 3.5 indicated very good taste (palatable taste).

2.2.8 Insent Taste Sensing System SA402B

Taste sensing system SA402B (Intelligent Sensor Technology, Atsugi, Japan), is the device to evaluate taste using sensors that mimic the human tongue. The Insent taste sensing system SA402B (figure 85) equipped with 8 sensors. Those 8 sensors with different characteristics evaluate the taste by using 2 kinds of sensor outputs, relative value (taste) and CPA value (aftertaste). Each sensor that responds selectively to one taste quality has been developed. So can see that various tastes can be evaluated by this system.

Figure 85: Insent Taste sensing system SA402B, (Intelligent Sensor Technology, Atsugi, Japan).

Response principle of taste sensing system is that lipid membrane played an important role in taste detection since twenty years ago, have been developed taste sensing system which mimics the living organism. Figure 86 shows response principle of the taste sensing system.

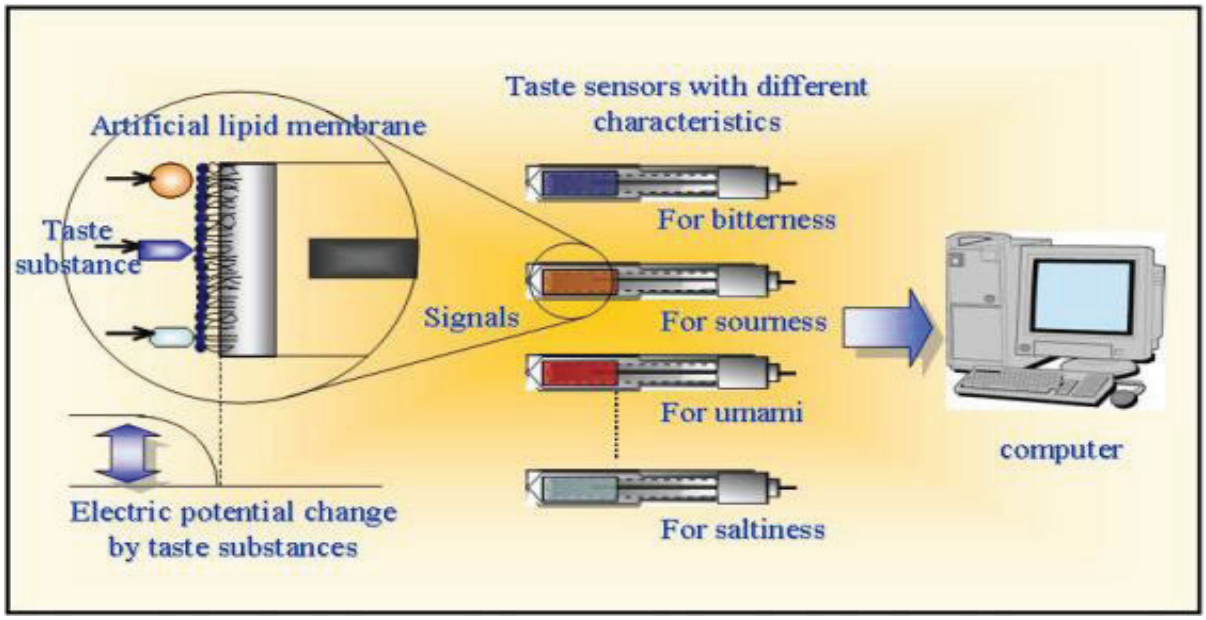


Figure 86: Response principle of the taste sensing system (Intelligent Sensor Technology, Atsugi, Japan).

The surface of each sensor is attached with artificial lipid membrane as an alternative to human tongue. The characteristics of eight sensors are different to each other. When these sensors are adsorbed with taste substances, the potential change of artificial lipid membrane occurs in the same manner as human tongue and personal computer calculates the data obtained from eight sensors and evaluate taste objectively.

In the figure 87, left photograph is taste sensor whose surface is attached with artificial lipid membrane. The sensors are comprised out of a sensor probe body and a terminal with an attached silver wire, which is plated with Ag/AgCl. Right photograph is reference electrode applying ceramics as junction part.

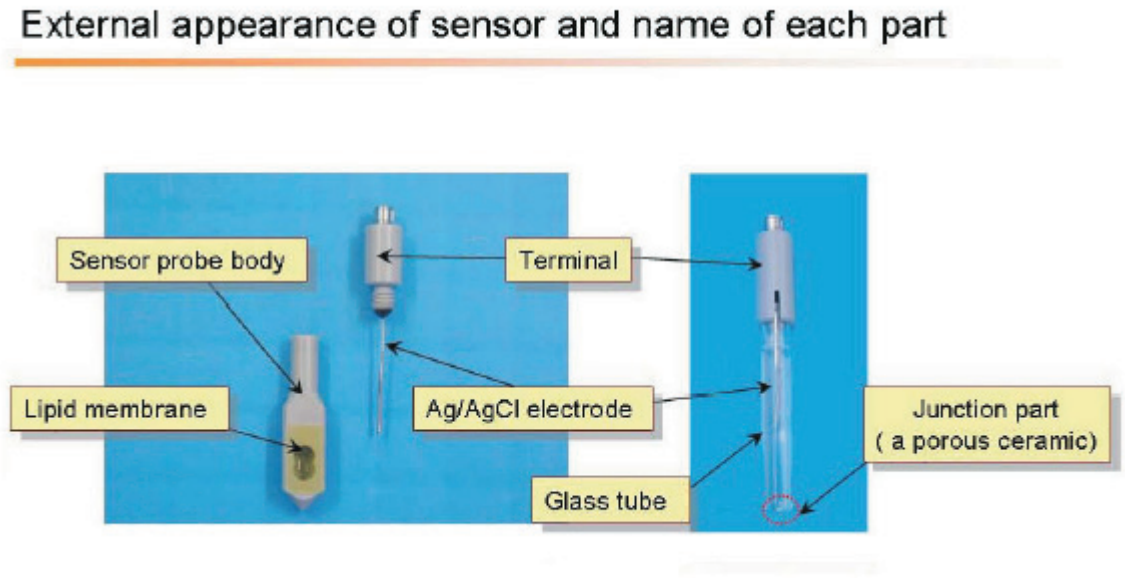
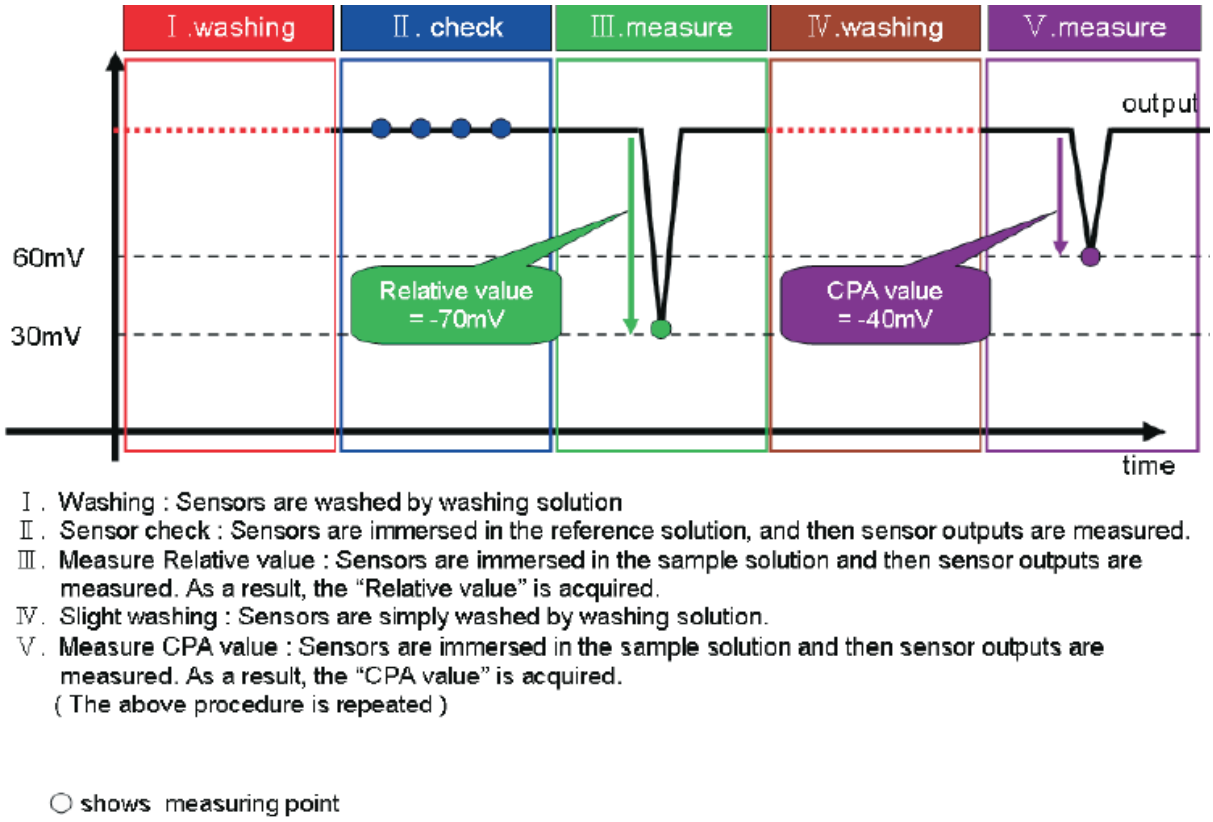


Figure 87: Taste sensor and reference electrode, Insent Sensors; left: Taste Sensor right: Reference electrode (Intelligent Sensor Technology, Atsugi, Japan).

The inner cavity, which is open to one side, is filled with 3.33 M potassium chloride (KCl) solution saturated with AgCl (inner solution) and closed by a lipid/polymer membrane. The reference electrode consists of the terminal part and a glass tube, which is also filled with inner solution, with a porous ceramic field at the tip (see figure 87).



Ⅰ. Washing : Sensors are washed by washing solution
Ⅱ. Sensor check : Sensors are immersed in the reference solution, and then sensor outputs are measured.
Ⅲ. Measure Relative value : Sensors are immersed in the sample solution and then sensor outputs are measured. As a result, the "Relative value" is acquired.
Ⅳ. Slight washing : Sensors are simply washed by washing solution.
Ⅴ. Measure CPA value : Sensors are immersed in the sample solution and then sensor outputs are measured. As a result, the "CPA value" is acquired.
(The above procedure is repeated)

○ shows measuring point

Figure 88: Measurement details (Intelligent Sensor Technology, Atsugi, Japan).

Before the test series, the stored sensors were filled with inner solution and left to saturate for half a day. The sensors were then attached to the sensor heads (2) of the system according to their membrane charge (positive or negative). A standard solution of 30 mM KCl and 0.3 mM tartaric acid and different washing solutions for sensors with positive and negative charged membranes were prepared and beakers with them positioned on the sampling rack. A sensor check was performed before each test series. The sensors were dipped repeatedly in standard solution as a cleaning procedure (90 s dipping into the first beaker set, 120 s each into the 2nd and 3nd set) and then conditioned in the standard solution for 30 s. None of the sensors should exceed the stability of 0.5 mV difference from the set value otherwise the procedure is repeated. Furthermore, all sensors should be in their respective mV range or should be replaced.

The measurement procedure of a sample followed a standard procedure, also displayed in figure 88. The first phase included the washing of the sensors for 30 s in three different beaker sets (the first with ethanolic washing solutions, the others with standard solution).

In the second phase the sensor output was conditioned for 30 s in the next set of standard solution, then the actual sample was measured for 30 s and the sensor outputs after this time recorded, afterwards the sensors were dipped shortly (30 s) in two standard solution beaker sets for cleaning. The last step was again the measurement of a standard solution similar to phase two. This procedure was repeated for every sample. Each sample was tested four times by rotation procedure (i.e., the first round of measurements of all samples was completed before the next round was started). The Insent sensors react, due to their specific lipid/polymer membrane, particularly to functional groups.

First, all sensors are immersed in standard solution and then reference value Vr are measured as initial sensor output. Standard solution is almost tasteless, so reference value Vr can also be regarded as information of tasteless.

Second, all sensors are immersed in sample solution. As a result, change of the membrane potential occurred and then sensor output Vr turns into Vs. and the value subtracting Vs from Vr is called relative value, which is equal to taste. For data analysis, mean relative values for each sample and sensor were calculated from the last three out of four runs.
Third, the sensors are shortly washed with standard solution and subsequently are immersed in the standard solution one again. In fact, when the sensors were immersed in sample solution, adsorptive substances such as astringent and bitter substances strongly adsorb the surface of lipid membrane, while ionic substances such as salty and sour substances are easily detached from the surface. Therefore, at the fourth stage, reference value Vr' (Vr prime) obtained in standard solution is different from reference value Vr at the first stage. The value subtracting Vr' (Vr prime) from Vr called CPA value. CPA is short for change of membrane potential caused by adsorption, which is equal to aftertaste.
Lastly, all sensors are washed with alcohol solution before measuring next sample.

In a process called “interpolating addition process”, the values obtained from the standard solution were used to define the zero point and all data from the samples were displayed in relation to it.

Sample data could be depicted in a radar plot or a PCA where the allocate on of the components could be chosen from the sensors. Further data analysis was possible with “transformation to taste information” by substituting the relative values into taste specific equations, through this process the mV data was changed into dimensionless “taste values”, table 40.
These values could again be displayed in either radar or PCA plots. If correlation with other data sets was required, a MRA data file was built through adding the additional data e.g. data from human taste panel results. The multivariate data analysis was then displayed in a PLS.

Table 40: Equations for the transformation of raw data into 8 kinds of taste information by the Insent Software (Intelligent Sensor Technology, Atsugi, Japan).

Taste information	Equation of taste information
Sourness	= 0.332 x sensor SB2CA0 + 12.0
Bitterness	= -0.140 x sensor SB2C00 + 0.084 x sensor SB2CT0
Adstringency	= -0.1575 x sensor SB2AE1 + 0.1575 x sensor CT0
Umami	= -0.1575 x sensor SB2AAE
Saltiness	= -0.252 x sensor SB2CT0
Aftertaste from bitterness	= -0.210 x CPA (C00)
Aftertaste from adstringency	= -0.252 x CPA (AE1)
Substance	= -0.420 x CPA (AAE)

2.3 Pellet production

2.3.1 Sieving

Powdered excipients were desagglomerated by sieving through a sieve with 1400 µm mesh size before further processing.

2.3.2 Blending of raw materials

After weighing the raw materials, the mixture was blended for 15 min at 30 rpm in a laboratory mixer (LM40, Bohle, Ennigerloh, Germany). Afterwards the mixture was transferred into the gravimetric powder feeder of the extruder (KT20, K-Tron, Soder, Lenzhard, Switzerland).

2.3.3 Extrusion

2.3.3.1 Wet extrusion

Powdered microcrystalline cellulose (MCC) or hypermellose (HPMC) with L-arginine free base or its salt monohydrochloride were fed from a gravimetric dosing device into the barrel of a co-rotating twin-screw extruder (Mikro 27GL-28D, Leistritz, Nürnberg, Germany) with a constant feed rate of 40 g/min and liquid feed rate was different depend on the formulations, 20 g/min for A and B formulations and 7 g/min for C and D formulations.

The granulation liquid was distilled water continuously pumped by a membrane pump (Cerex EP-31, Bran und Lübbe, Norderstedt, Germany) with a flow through metering device (Corimass MFC-081/K, Krohne, Duisburg, Germany). During process time the powder and liquid feed rate, screw speed, driving power, temperature and pressure at the screen plate was recorded. The mass was extruded through a die plate with 23 dies of 1 mm diameter and 2.5 mm length for all experiments. They were extruded at a constant screw speed of 30 rpm. The formulations were extruded at room temperature 25°C.

2.3.3.2 Lipid extrusion

Powdered lipids of different chemical compositions or mixtures of these lipids with 50% of L-arginine free base or its salt monohydrochloride were fed from a gravimetric dosing device into the barrel of a co-rotating twin-screw extruder (Mikro 27GL-28D, Leistritz, Nürnberg, Germany) with a constant feed rate of 40 g/min.
The mass was extruded through a die plate with 23 dies of 1 mm diameter and 2.5 mm length for all experiments. They were extruded at a constant screw speed of 30 rpm. Material temperature was measured next to the die plate just before the extrusion step. In all experiments carried out for lipid/drug mixtures were extruded successively at different cylinder temperatures. At each temperature level the different cylinder segments were tempered at the same cylinder temperature. Five batches of two formulations were extruded by using different extrusion conditions (table 25). The extruder was loaded with the lipid/drug mass, the screw transport was stopped and the cylinder segments were heated to 100°C.

After a 20 min tempering period the cylinder segments were cooled down to 50°C and the lipid/drug mass was extruded through the die plate.

2.3.3.3 Evaluation of extrudates and pellets

The extrudates and pellets were visually analyzed for any apparent defects: shark-skinning, cracks, hairlines, curling or deformation by melting. The absence of defects gave information about the process conditions of good extrudability and spheronizability.

2.3.4 Spheronization

The Schlueter spheronizer (RM 300, Schlueter, Neustadt/ Ruebenberge, Germany), was used for shaping granules into pellets. Extrudates of every batch about 300 g were transfer to the spheronizer to get pellets with spherical shape by using for every batch different spheronization conditions. The apparatus was equipped a friction plate had a diameter of 300 mm. The surface area of the friction plate was cross-hatched (see figure 89 for a detail drawing). Compressed air was used to keep the gap between rotor plate and spheroniser jacket open. The speed of the rotor plate, jacket temperature and spheronisation time were varied for the spheronisation of lipid formulations and are explained in detail in (table 26). The friction plate and the spheronizer wall were cleaned after each batch to guarantee the same spheronizer conditions.

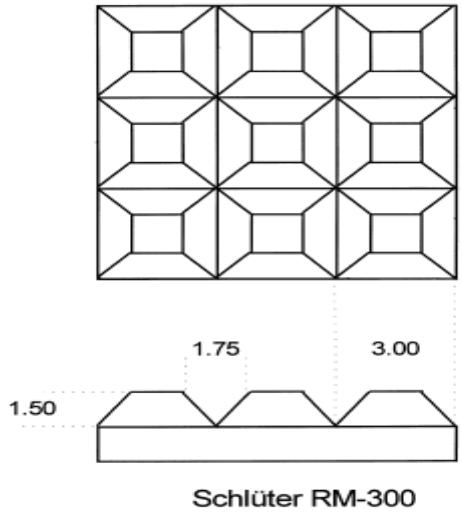


Figure 89: Schematically drawings of the friction plates of Schlüter RM-300 spheronizer [92].

2.4 Pellet Characterisation

2.4.1 Yield

Sieve analysis was carried out for each batch in order to remove fines and agglomerates. A set of sieves (Retsch, Haan, Germany) was chosen ranging from 0.8 mm to 2 mm (Retsch, Haan, Germany).

The sieving was performed with a vibrio (AS200 Control, Haan, Germany) for 3 min at amplitudes of 2. The weight of pellets retained on each sieve was recorded and presented. The fraction of pellets with diameters between 0.8 mm and 2 mm was defined as yield. Using a rotary cone sample divider, suitable samples from the yield fraction were obtained (Retschmuehle PT, Retsch, Haan, Germany).

2.4.2 Pellet shape, size and distribution

Image analysis was conducted using a system consisting of a stereo microscope (Leica MZ 75, Cambridge, UK), a ring light with cold light source (Leica KL 1500, Cambridge, UK), a digital camera (Leica CS 300 F, Cambridge, UK) and image analyzing software (Qwin, Leica, Cambridge, UK).

At least 500 pellets from each batch of the yield fraction were analyzed at a suitable magnification (1 pixel = 8.75 µm) and translated into binary images. Contacting pellets were isolated from the data processing using a software algorithm or deleted manually if the automatic function of separation failed. For each pellet, 64 Feret diameters and the projected area were determined. The pellet size and shape were characterized by equivalent diameter and aspect ratio, respectively.

Shape

The aspect ratio is used as a method to characterise the shape of the obtained pellets. It is defined as the ratio between the maximum (d_{max}) and the Feret-diameter perpendicular to it ($d_{90°}$) (Eq.5):

$$AR = \frac{d_{max}}{d_{90}} \qquad \text{Eq. (5)}$$

Size and size distribution

The pellet size is described by the equivalent diameter (d_{eq}), which is calculated on the basis of a projected pellet surface (A) [93] (Eq. 6):

$$d_{eq} = \sqrt{\frac{4A}{\pi}} \qquad \text{Eq. (6)}$$

To characterize the similarity of the pellet size distribution, [94] normalized every pellet diameter (d) to the median (d_{50}) of each distribution. The result is a dimensionless diameter (d_d) (Eq. 7):

$$d_d = \frac{d_{eq}}{d_{eq50}} \qquad \text{Eq. (7)}$$

Where d_d is the dimensionless diameter, d_{eq} the equivalent diameter and d_{eq50} the median of all equivalent diameters, the distribution of the particle size was characterised by the fraction of the particles in the interval $0.9 < d < 1.1$. The size distribution was characterised as “good” if the fraction of this 10 % interval exceeded 50 % and very good if the fraction of this 10 % interval exceeded 90% [95].

2.4.3 Helium pycnometry density

The density of each pellet batch was measured by gas pycnometric density (ρ_{He}) with helium as test gas at 25°C (AccuPyc 1330TC Pycnometer, Micromeritics, Norcross, GA, USA). The pellets of each batch were transferred into a sample chamber of a volume of 10 cm^3 with the filling and equilibration pressure of 134 kPa and were flushed tenfold with helium. The density was determined as average value of five single measurements. For each tested pellet batch, three samples were analysed.

2.4.4 Mercury porosimeter density

Mercury porosimeter density of each pellet batch was determined using a PASCAL 140 mercury porosimeter (Thermo Finnigan Italia S.p.A., Rodano, Italy) on the same samples of pellet were used before for the helium density.

Approximately 2 g sample of each batch in a dilatometer (type CD3P) was evacuated for 15 min. After evacuation and filling of mercury into the dilatometer, volume determination was done automatically.

2.4.5 Porosity

The Porosity ε of each pellet batch was calculated using the helium and mercury density according to following equation:

$$\varepsilon = 1 - (\text{mercury porosimeter density} \ / \ \text{pycnometric density}) \qquad \text{Eq. (8)}$$

2.4.6 Storage stability studies

Samples of lipid pellets were hermetically packed and stored at stressed conditions with an elevated temperature of 40°C in a drying oven (ET 6130, Heraeus, Hanau, Germany) for 6 months, to observe changes in release profiles through thermal treatment of lipid pellets. Dissolution studies and DSC analyses were performed after the storage period.

2.4.7 In vitro drug dissolution studies

The dissolution medium (50 mM phosphate buffer at a pH of 6.8) was prepared as follows. Initially 20.0 ml of 10 M NaOH were pippetted into a 1000 ml volumetric flask and diluted with purified water to prepare 0.2 M NaOH as a stock solution.

Subsequently 54.44g of Potassium Dihydrogen Phosphate, anhydrous was weighed into a suitable container, and dissolved and diluted with 2000 ml purified water. 896 ml of the 0.2 M NaOH was added to the container and diluted to 8000 ml with purified water. Finally the mixture was degassed before use. In addition to using phosphate buffer as a dissolution media, another two dissolution media were used, purified water and 0.1 N hydrochloric acid. The dissolution tests were performed by using the Ph. Eur. basket apparatus method at 75 rpm (Pharma Test PTW S III, Pharma Test Apparatebau, Hainburg, Germany)

Where, 2 g of the pellets of each batch were placed in each vessel of the dissolution apparatus equipped with 6 vessels that contain 1 litre of the dissolution medium for immediate rotation at 75 rpm at about 37° C.±0.5°C.

Basket setup was used; about 200 µL of the solution from each vessel was removed at different time points for respective dissolution analysis at each time point. Each of these samples solutions were filtered through 0.45 µm polypropylene membrane syringe filter. The filtrate was collected into HPLC vials for analysis.
HPLC was used to determine the concentration of L-arginine in the dissolution media by measuring the absorption at 210 nm. Preliminary investigations showed that the formulation aids do not have an impact on the absorption at the wavelength 210 nm. All experiments were conducted in five replicates. Validation of the analytical HPLC method for the determination of L-arginine concentration was performed.

2.4.8 Differential scanning calorimetry

Thermal characteristics of the powdered lipids and the pellet samples before and after stored at a defined storage time were studied with a Mettler DSC 821e (Mettler Toledo, Giessen, Germany). DSC scans were recorded at a heating rate of 5°C/min. Samples with an initial weight of approximately 4 mg were heated from 20 to 200°C in a sealed and pierced aluminium pan. Data analysis was performed with the STAR^e program Version 6.01 (Metter-Toledo, Giessen, Germany). An empty aluminium pan was used as reference.

2.4.9 Scanning electron microscopy

For scanning electron microscopy, samples were fixed with conductive silver on brazen specimen holders. Gold sputtering was conducted with an Agar Sputter Coater (Agar Scientific Ltd., Stansted, Essex, England), each for 60 s under vacuum. Between the sputtering intervals a lag of time of 15 s was inserted to avoid an overheating of the samples. The vacuum was kept constant at the whole time. The samples of every batch were observed by using a scanning electron microscopy (LEO VP 1430, Carl Zeiss NTS GmbH, Oberkochen, Germany). During the measurement the high voltage of the cathode was 10 kV and an integration of 50 images of one adjustment was used to get one picture.

2.4.10 Pellet preparation

In an evaporating dish samples of each batch were putted and immersed in liquid nitrogen. Afterwards the nitrogen was evaporated. By using a pestle, the frozen pellet samples were tapped on. On the brass specimen holder, fragments of split pellet samples were attached for scanning electron microscopy.

2.4.11 Raman spectroscopy

Raman spectra were obtained from solids in a capillary by using a BIO-RAD Excalibur spectrometer kit (Bio-Rad Laboratories, Cambridge, MA), equipped with the excitation beam is a Nd:YAG laser Raman accessory (yttrium aluminium garnet crystal doped with triply ionised neodymium) (Spectra Physics, Mountain View, CA) with an output in the near infrared at 1064 nm operating at power level of 450 mW. Preliminary Raman spectra were registered at power level 50 mW. The samples are respectively presented to the spectrometer in glass tubes of an internal diameter of 12 mm and a length of 75 mm, and in glass tubes of an internal diameter of 5 mm and a length of 75 mm. These tubes are introduced into a dedicated thermostatic sample holder made of aluminium to enhance the collection of the scattered light. The sample holder is placed in the sample compartment. Raman shift was calibrated by a silicon slide at 520 cm^{-1}.

The spectra (130-2200 cm^{-1}) were collected at room temperature in the dark under the following conditions: 100 mW of laser power, 2 cm^{-1} resolution, 30 s exposure times, and 10–20 scans. Spectral data were baseline corrected using the GRAMS/32 AI Software (Galactic Industries Corporation, Salem, NH).

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