Evaluation of various betaine sources and investigations on the stability of vitamins in dependence on the supplementation of choline chloride or different betaine sources

D. Kampf[1], S. Mangelinckx[2)*], N. De Kimpe[2], und L. Segers[3]

[1] Orffa Additives B.V., Werkendam, The Netherlands
[2] Ghent University, Department of Sustainable Organic Chemistry and Technology, Faculty of Bioscience Engineering, Gent, Belgium, * Postdoctoral Fellow of the Research Foundation - Flandern (FWO), Belgium
[3] Orffa Belgium N.V., Bornem, Belgium

Introduction

Betaine is used mainly in animal nutrition as a methyl group donor and may be used to replace choline chloride, which is generally regarded as hygroscopic and difficult to handle. In the past, betaine was obtained exclusively from sugar beet, today however, various chemically bound forms from different manufacturing processes are available. The biological properties (bioactivity) of the different origins and production methods are frequently debated, although on the effect of betaine numerous publications are available (Eklund *et al.* 2005, Ratriyanto *et al.* 2009). In particular, the osmoregulatory properties of chemically synthesized betaine hydrochloride compared with those produced from natural betaine sources i.e. sugar beet are in question. On the other hand also betaine monohydrate and anhydrous are criticized for being very hygroscopic, and thus negatively influencing the stability of feed.

Biological value of various Betaine sources

The aim of the first study was to evaluate the biological value of various betaine sources (hydrochloride, monohydrate and anhydrous), because in this respect the question is often asked whether osmoregulatory properties of betaine hydrochloride are equal betaine anhydrous. To investigate this question, an *in vitro* study was carried out in which a total of 4 samples were studied by simulating stomach conditions (betaine monohydrate or betaine anhydrous from sugar extraction (MonoEx or AnhydEx respectively), betaine hydrochloride or betaine anhydrous from chemical synthesis (HydroSyn or AnhydSyn respectively)). The samples (10 mg) were dissolved in 10 ml H_2O, adjusted with a 37% HCl solution to pH 2.3 and pH 4.5 and subsequently analyzed by LC-MS and direct MS. The mass spectrometry was selected as a well defined chemical analysis because it is ideal for obtaining an insight into the chemical structure of a molecule. The mass spectra were determined on the one hand on an Agilent 1100 system with a Phenomenex Luna C18 column and DAD, in this case, the flow rate of 1 ml/min with 5 mM NH_4OAc solution in water-acetonitrile was adjusted as the mobile phase. For direct MS an Agilent 1100 system was used with direct injection.

Results

At pH 2.3 betaine was measured in the LC-DAD (220 nm) for all four samples after about 2.7 min. In the positive electro spray ionization mode of the total ion chromatogram (TIC) the protonated betaine molecules $[M+H]^+$ at *m/z* 118 (*m/z* retention time of approximately 2.7 min, figure 1) and also the cluster $[2M+H]^+$ at *m/z* 235 showed for all four samples a high intensity. In the negative electro spray ionization mode of the total ion chromatogram the ions $[M+Cl]^-$ at *m/z* 152/154 showed, on the other hand, only a low intensity. Similar results were obtained in the direct MS analyzes (table 1).

Based on these data it can be concluded that betaine dissolved in acidic solutions (pH 2.3) leads to similar quantitative results by LC-MS analysis.

Table 1: Retention times of various sources of betaine in the LC-MS and direct MS at pH 2.3

Sample	LC-MS Ret. time min	LC-MS ES, pos. Mode *m/z* (%)	LC-MS ES, neg. Mode *m/z* (%)	Direct MS ES, pos. Modes *m/z* (%)	Direct MS ES, neg. Mode *m/z* (%)
MonoEx	2.69	235 (100) 118 (52)	176 (100) 152/154 (17)	235 (100) 118 (51)	176 (100) 152/154 (95)
AnhydEx	2.69	235 (100) 118 (34)	152/154 (100)	235 (60) 118 (100)	169/171/173/175 (32) 152/154 (100) 120 (59)
HydroSyn	2.70	235 (100) 118 (27)	152/154 (100)	235 (100) 118 (44)	684 (70) 152/154 (100)
AnhydSyn	2.67	235 (100) 118 (24)	299 (58) 176 (36) 152/154 (100)	235 (100) 118 (20)	169/171/173/175 (100)

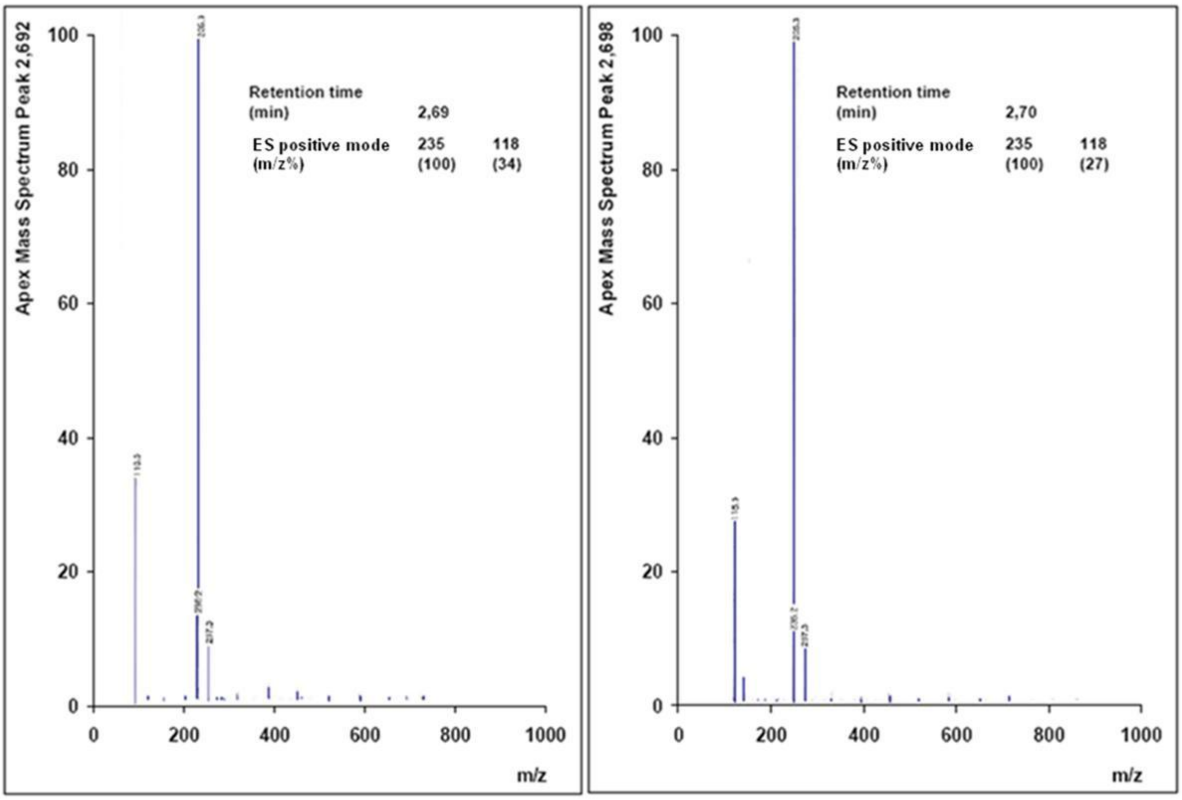
Betaine at pH 4.5 showed in the positive electro spray ionization mode of the total ion chromatogram for both the protonated molecules $[M+H]^+$ (*m/z* retention time of about 2.7 min) at *m/z* 118 and for the cluster at *m/z* 235 $[2M+H]^+$ and *m/z* 257 $[2M+Na]^+$ a high intensity (table 2).

Based on these data it can be concluded that betaine dissolved in acidic solutions (pH 4.5) also leads to similar quantitative results by LC-MS analysis.

Table 2: Retention times of various sources of betaine in the LC-MS at pH 4.5

Sample	LC-MS Ret. time min	LC-MS ES, pos. Mode *m/z* (%)
MonoEx	2.,66	257 (23), 235 (100), 118 (44)
AnhydEx	2.67	257 (20), 235 (100), 118 (35)
HydroSyn	2.67	257 (24), 235 (100), 118 (38)
AnhydSyn	2.65	257 (40), 235 (100), 118 (51)

The results show that betaine molecules after simulated gastric passage (pH 2.3 and pH 4.5) are presented independent of the chemical bonding form and type of production (extraction from sugar beet or chemical synthesis) as identical molecules in dissociated form (same *m/z* retention times in the LC-MS and MS), which is considered as evidence that the betaine binding form and the type of the production does not affect the biological activity and osmoregulatory function.



Mass Spectrum Betaine Anhydrous (sugar beet extraction) **Mass Spectrum Betaine Hydrochloride (Chemical synthesis)**

Figure 1: LC-MS analysis of betaine anhydrous and betaine hydrochloride

Stability of vitamins in dependence of the supplementation of choline chloride or different sources of betaine

In the second study betaine hydrochloride, choline chloride or betaine anhydrous were added to a premix for piglets (dosage 0.5% in the diet) and the influence of a storage of up to 6 months (measured at 0, 0.5, 1, 3 and 6 months) was examined for the change in the dry matter content as well as the degradation of vitamin A (tables 3 and 4). The products tested were choline chloride 60% on an organic carrier with 52% choline and betaine hydrochloride with 70.9% betaine and betaine anhydrous with 93% betaine.

Table 3: Calculated concentrations of choline and betaine in the premixes

		Control	Choline chloride	Betaine hydrochloride	Betaine anhydrous
Choline chloride	mg/kg premix	0	150.000	0	0
Betaine	mg/kg premix	0	0	125.859*	125.859*

* Levels resulting from the calculation of the equivalent concentration based on the molecular weights of choline chloride (139.6 g/mol) and betaine (117.2 g/mol)

The selection of the vitamin to be tested was partly based on the Shurson *et al.* (2010) proposed rankings, it was also taken into account what vitamins are critically evaluated by premix manufacturers and what vitamins are most common analysed in official investigations. The analysis of the vitamin contents in the premix were performed according to standard procedures for vitamins in premixes (analysis latitude 5%) by an independent laboratory (Oleotest, Lageweg 427, 2660 Antwerp, Belgium). The hygroscopicity measurements were performed in the laboratory of the premix manufacturer (Vitamex, Booiebos 5, 9031 Drongen, Belgium) and the method applied corresponded to the standard laboratory analysis for measuring hygroscopicity. Air at 80% relative humidity flowed through a saturated ammonium chloride solution and then through the matrix to be examined at a constant storage temperature of 20oC and 37oC, the test material was then weighed every two hours until a constant final weight was reached. The results were calculated as follows and illustrated:

= (% Weight gain + % free water) / (100 + % increase in weight) * 100 Provision of free water by 6-hour drying at 87°C

Rating scale results:

<10.0%	virtually non-hygroscopic
10.1 to 15.0%	low hygroscopic
15.1 to 20.0%	moderately hygroscopic
20.1 to 25.0%	very hygroscopic
> 25.0%	extremely hygroscopic

Table 4: Composition of the premix

Additive	Raw material	Premix mg/IU/FTU per kg	Feed mg/IU/FTU per kg
Copper	Copper sulphate Pentahydrate	3,000	15
Iron	Iron sulphate Monohydrate	24,000	120
Zinc	Zinc sulphate	20,000	100
Manganese	Manganese oxide	16,000	80
Selenium	Sodium selenite	70	0.35
Iodine	Calcium iodate	300	1.5
Vit A	Vit A 1000	3,000,000 IU	15,000 IU
Vit D3	Vit D3 500	400,000 IU	2,000 IU
Vit E	Vit E 50% Adsorbat	20,000	100
Vit B1	Vit B1 81%	300	1,5
Vit B2	Vit B2 80%	1153	5.76
Vit B3	Vit B3 98% D-Calpan	3,500	17.50
Vit B6	Vit B6 100%	600	3.0
Vit B12	Vit B12 0,1%	9	0.045
Vit Niacin	Vit Niacin 100%	5,600	28
Vit K3	Vit K3	300	1.5
Phytase	3-Phytase EC3.1.3.8 4ª1600	100,000 FTU	500 FTU
BHT		170	0.85
Ethoxyquin		53	0.27
Propylgallaat		53	0.27
Carrier: Lime stone, Wheat bran			

Results

The analysed levels of choline and betaine show that dependent of a storage time of up to 3 months a reduction of up to 20% has to be assumed. An influence of storage temperature, however, was not observed. A clear trend in differences in stability between the different products studied here from this research approach could not be derived (table 5).

Table 5: Stability of choline chloride and betain in dependence storage time and temperature

		Control	Choline chloride	Betaine hydrochloride	Betaine anhydrous
calculated	mg/kg premix	0	150,000 (100)	125,859 (100)	125,859 (100)
t 0	mg/kg premix	< 1.000	136,000 (91)	118,000 (94)	121,000 (96)
t 1 mon 20°C	mg/kg premix		125,000 (83)	114,000 (91)	117,000 (93)
t 1 mon 37°C	mg/kg premix		124,000 (83)	98,000 (78)	106,000 (84)
t 3 mon 20°C	mg/kg premix		122,000 (81)	97,700 (78)	95,200 (76)
t 3 mon 37°C	mg/kg premix		121,000 (81)	102,000 (81)	99,000 (79)

() Values relative to the calculated value

The storage period had a significant influence on the degradation of vitamin A in premixes (table 6). This was, for example, in the control group after 6 months (20°C storage temperature) approximately 25%, as well as in the premixes containing choline chloride or betaine anhydrous. Remarkable was the much smaller decrease of about 10% in the premix containing betaine hydrochloride. The influence of the storage temperature was also difficult to see here. Only in the premix containing choline chloride significantly lower levels of vitamin A could be detected after 3 and 6 month in contrast to the other experimental groups.

Table 6: Vitamin A stability in the premix in dependence of storage time and temperature as well as the supplementation of choline chloride and various betaine sources

		Control	Choline chloride	Betaine hydrochloride	Betaine anhydrous
calculated	UI/kg* premix	3,000 (100)	3,000 (100)	3,000 (100)	3,000 (100)
t 0	UI/kg* premix	3,206 (107)	3,340 (111)	3,275 (109)	3,067 (102)
t 0.5 mon 20°C	UI/kg* premix	3,053 (107)	3,005 (100)	3,188 (106)	2,916 (97)
t 1 mon 20°C	UI/kg* premix	2,782 (93)	2,901 (97)	3,027 (101)	2,835 (95)
t 3 mon 20°C	UI/kg* premix	2,334 (78)	2,491 (83)	3,021 (101)	2,440 (81)
t 6 mon 20°C	UI/kg* premix	2,220 (74)	2,263 (75)	2,713 (90)	2,342 (78)
t 0.5 mon 37°C	UI/kg* premix	2,839 (95)	2,959 (99)	3,166 (106)	2,778 (93)
t 1 mon 37°C	UI/kg* premix	2,761 (92)	2,770 (92)	2,649 (88)	2,457 (82)
t 3 mon 37°C	UI/kg* premix	2,330 (78)	1,986 (66)	2,605 (87)	2,395 (80)
t 6 mon 37°C	UI/kg* premix	2,316 (77)	1,613 (54)	2,532 (84)	2,382 (80)

() Values relative to the calculated value UI/kg* = 1.000 UI/kg

The individual added feed additives and especially the supplementation of choline chloride and betaine anhydrous significantly influenced the hygroscopic properties of the respective premixes (table 7). The measured moisture values for these additives were with about 12.0% far higher than by the use of betaine hydrochloride (7.5%) or in the control group (3.3%) and thus demonstrate the high risk of adverse hygroscopic properties of these products. In addition, this can at least partially explain the increased degradation rate of vitamin A by use of choline chloride, suggesting that it seems to be a combined negative effect of hygroscopicity and reactivity.

Table 7: Water absorption behaviour of premixes in dependence of the supplementation of choline chloride and various betaine sources

		Control	Choline chloride	Betaine hydrochloride	Betaine anhydrous
Water absorption	%	3.3	12.01	7.52	11.79

Summary

Betaine is widely used as feed additive in animal nutrition, where it is used primarily as a substitute for choline chloride, which is generally considered hygroscopic and difficult to process. Betaine is available in various chemically bound forms from a variety of manufacturing processes, although the biological properties (bioactivity) of the individual origins and production methods are still controversially debated to date.

In the studies presented here could be shown that several sources of betaine in LC-MS analyses provide identical results, after which of conclusion, the biological activity of each betaine source can be equated.

In our experiment, where the stability of premixes containing vitamin A was observed, it was demonstrated, that a storage time of 6 months (normal storage temperature) is accompanied by a reduction in vitamin A of about 25%. Only in the premix with betaine hydrochloride was a significantly smaller decrease of only about 10% demonstrated. An influence of an elevated storage temperature could only be found after 3 and 6 months in the premix that contained choline.

With regard to the hygroscopic properties especially the supplementation of choline chloride and betaine anhydrous affected the stability of the respective premixes, the measured moisture values were up about 12.0%, much higher than with the use of betaine hydrochloride (7.5%) or in the negative control group (3.3%).

References

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Authors address

Dr. Detlef Kampf
Orffa Additives B.V.
Vierlinghstraat 51
4251 LC Werkendam
The Netherlands
e: kampf@orffa.com